The Insulin-like Growth Factor (IGF) system in breast and colorectal carcinogenesis: dietary intervention and molecular studies

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The Insulin-like Growth Factor (IGF) system in breast and colorectal carcinogenesis: dietary intervention and molecular studies

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door

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geboren te Smilde

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HET KAN GEBEUREN DAT DE strijder midden in het eindeloze strijdgewoel plots een idee krijgt, waarna hij in een oogwenk de strijd weet te beslechten.
En hij denkt: waarom heb ik zo moeten ploeteren, het gevecht had maar half zo lang hoeven duren.
Eenmaal opgelost, lijkt een probleem altijd heel eenvoudig. Een grote overwinning, die achteraf gemakkelijk lijkt, is steeds het resultaat van een reeks kleine, vrijwel onopgemerkte overwinningen.
Dan begrijpt de strijder wat er is gebeurd, en slaapt vervolgens heerlijk. In plaats van het zichzelf kwalijk te nemen dat hij er zo lang over heeft gedaan, is hij blij dat hij het heeft geklaard.

Uit: Paulo Coelho, De strijders van het licht, 2002

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GENERAL INTRODUCTION

1

Breast and colorectal cancer

Each year, 10.9 million people worldwide are diagnosed with cancer. After lung cancer, breast cancer is the second and colorectal is the third most common cancer with 1.2 million (11%) and 1.1 million (9%) incident cases per year, respectively (1). Figures from the Netherlands show that in 2003, 11,800 of the 35,500 (32.7%) incident cancer diagnoses in women were attributable to breast cancer (2). With respect to colorectal cancer, 4,740 (13.3%) incident cases were observed among women, and 5,160 (13.8%) among men. These numbers are comparable with those observed in other Western European countries and Northern America. The incidence of breast cancer is now leveling down, whereas colorectal cancer incidence is still increasing (2). More than one out of three individuals in the Netherlands will develop cancer during their life; one out of 8 women will get breast cancer, and one out of 18 women and one out of 16 men will develop colorectal cancer (2).

Carcinogenesis

The development of breast and colorectal cancer is a multistep process, in which the accumulation of different mutations in somatic cells results in the transition from normal breast and colon epithelium to premalignant lesions (i.e. intraepithelial neoplasia, adenoma) and eventually (invasive) carcinomas (**Figure 1.1**).

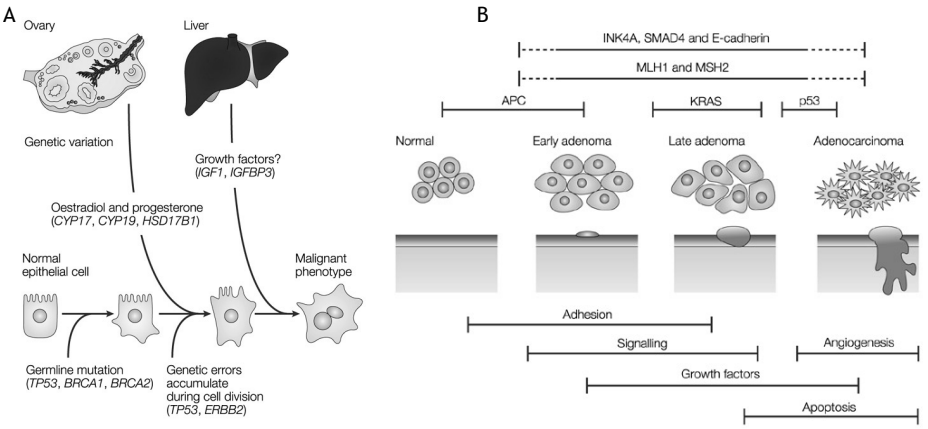

Figure 1.1. Molecular events that characterize the transition of normal epithelial breast (A) or colorectal (B) cells to a malignant phenotype. From Kolonel et al. (3) and Kerr et al. (4).

Inherited factors explain about 5% of all breast and colorectal cancers and strongly predispose to the development of these cancers. These include germline mutations in BRCA1, BRCA2, and p53 for breast cancer, and germline mutations in the APC gene (Familial Adenomatous Polyposis) and mismatch repair genes, e.g. MLH1 and MSH2 (Hereditary Non-Polyposis Coli), for colorectal cancer.
However, the majority of cancers occurs sporadically and is associated with somatic

genetic mutations acquired during life (Figure 1.1). The development of these mutations is caused by inherited factors (e.g. genetic susceptibility), environmental and / or lifestyle factors, or an interaction between these factors. Exposure to hormonal and growth factors, such as the insulin-like growth factors (IGF), may also influence this process.

Cancer prevention

In 1981, Doll and Peto published a landmark study of the causes of cancer that was based, in part, on a review of cancer incidence across many countries (5). They concluded that 75-80% of cancers in the US in 1970 might theoretically have been prevented by altering environmental factors (smoking, alcohol consumption, diet) (5). Although they acknowledged this estimate was a theoretical maximum, their analysis provided an important starting point for further studies of cancer causes and strategies for cancer prevention. In the past 25 years, subsequent studies extended the list of risk factors, to include obesity and lack of physical activity. Specifically, it is estimated that achievable changes in the preventable causes of cancer now account for more than 50% of all cancer cases in the US. Epidemiological evidence indicates that in the US, diet accounts for 10-25% of all cancer cases (6). Established and potential risk factors for breast and colorectal cancer are shown in **Table 1.1**.

Potential endpoints for cancer chemoprevention

The different stages of carcinogenic progression might depend on different molecular abnormalities or signaling pathways (Figure 1.1). Targeting specific abnormalities is suggested to be most effective during the earlier, premalignant phases of carcinogenesis, at which time there are minimal abnormalities present (7). Additionally, randomized chemoprevention trials using cancer as an endpoint are generally large, lengthy and costly. Therefore, studies with surrogate endpoints (biomarkers of preclinical carcinogenesis) are very attractive, and are potentially smaller, shorter and considerably less expensive than those with cancer endpoints (8). Studies using surrogate endpoints are mostly performed in high-risk individuals who do not yet have cancer. However, they are inherently less reliable than studies with the 'true' endpoint (8). Examples of surrogate end points are histological, cytological and genomic abnormalities in histologically normal epithelium indicative of apoptosis, proliferation, differentiation and transformation (e.g. Ki-67, PCNA, intraepithelial neoplasia, growth factors), and molecular markers in blood and tissues (e.g. oxidation products and antioxidants, hormones, and growth factors including the insulin-like growth factor) (8;9).

Table 1.1. Established and potential risk factors for breast and colorectal cancer (10-14)

Breast cancer	Colorectal cancer
Established risk factors	
• Age • Mammographic breast density / benign breast disease • Personal or family history of breast cancer • Hereditary conditions *BRCA1 and BRCA2* • Lack of physical activity • Overweight *Postmenopausal women* • Reproductive factors *Early menarche, nulliparity / late first birth, late menopause, no / short breastfeeding* • Exogenous hormone use *Hormone replacement therapy, oral contraceptive use, diethylstilbestrol* • Alcohol consumption • Exposure to ionising radiation	• Age • Personal history of colorectal polyps • Personal or family history of colorectal cancer • Hereditary conditions *Familial Adenomatous Polyposis, Hereditary Non-Polyposis Coli* • Inflammatory diseases of the colon and rectum • Lack of physical activity • Overweight • Regular use of aspirin and other NSAIDs *Decreased risk* • Alcohol consumption • Diet *Red meat, processed meat*
Potential risk factors	
• Diet *Decreased risk for soy products*	• Smoking • Diet *Decreased risk for fruits, vegetables, fiber, folic acid, calcium, vitamin D* • Exogenous hormone use *Decreased risk for hormone replacement therapy, oral contraceptive use* • Diabetes

The IGF-system

The primary role of the Insulin-like Growth Factor (IGF)-system is the regulation of prenatal and postnatal growth (15;16). However, it has also been involved in carcinogenesis (17). The IGF-system consists of two ligands, IGF-I and IGF-II; two cell-membrane receptors, IGF-I receptor (IGF-IR) and IGF-II receptor (IGF-IIR); and six high-affinity IGF binding proteins, IGFBP-1 through –6 (18).

IGF-I is predominantly produced by the liver, and its synthesis is regulated by both hormonal (e.g. growth hormone, estradiol) and nutritional factors (e.g. dietary energy and protein intake). The key stimulator of IGF-I production is growth hormone (GH), which is produced in the pituitary gland under control of the hypothalamic factors growth-hormone-releasing hormone (GHRH) and somatostatin (SMS) (**Figure 1.2**). IGF-II is also produced by the liver, but is not tightly regulated by GH (18).

In the blood circulation, about 90% of IGF-I is bound in a complex with IGFBP-3 and acid-

labile subunit (ALS), which are also derived from the liver (Chapter 2, **Figure 2.1**). This complex is too large to cross the capillary epithelium. However, IGF-I can also bind to other IGFBPs, of which IGFBP-1 and –2 are most well described. When IGF-I is bound to these two IGFBPs, or when it is in its free from (<1%), it can be transported out of the blood stream to specific target tissues (18).

IGFBPs have a very important although dual role in regulating the bioavailabilty of IGF-I and –II (18). IGFBPs prolong the half-life of IGFs and when they are degraded by proteases, they render free IGFs to interact with the IGF-IR. The IGF-IR is a tyrosine kinase cell-surface receptor and can bind either IGF-I or IGF-II. Following ligand binding to IGF-IR, its tyrosine kinase activity is activated, and this stimulates signaling through intracellular networks. Key downstream networks include the phosphatidylinositol 3'-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathway. Activation of these pathways stimulates proliferation and inhibits apoptosis (18). On the other hand, IGFBPs may inhibit IGF-I signaling by competing with the IGF-IR for available IGFs. IGFBP-1, -3 and -5 also have direct, IGF-independent growth regulatory actions (19). IGF-II can also be bound by IGF-IIR, whereby the amount of available IGF-II for binding to IGF-IR is reduced (18).

Besides being derived from an 'endocrine' source, IGFs and IGFBPs can be locally produced through autocrine or paracrine mechanisms. These mechanisms often involve interactions between stromal and epithelial cell subpopulations (Figure 1.2).

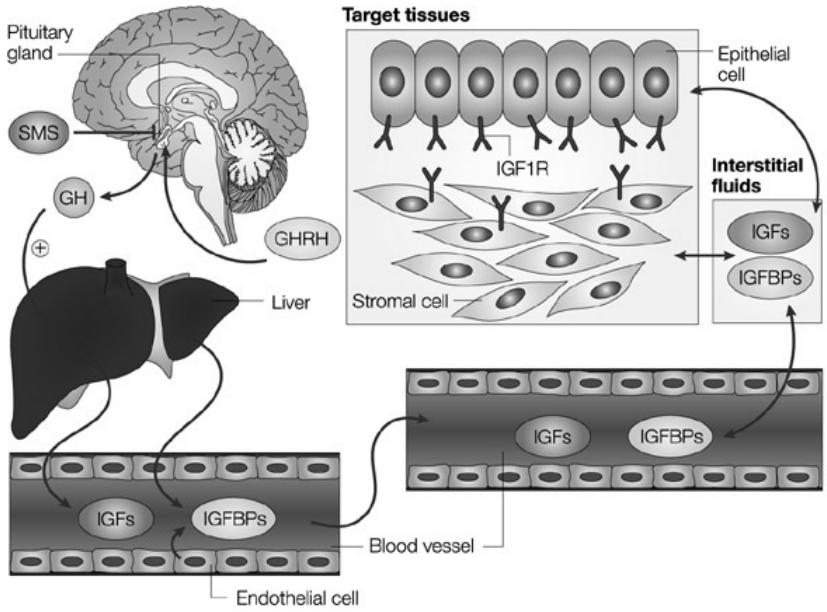

Figure 1.2. The IGF-system; regulation of circulating and tissue levels of insulin-like growth factors. From Pollak et al. (18). GH, growth hormone; GHRH, growth-hormone-releasing hormone; SMS, somatostatin; IGFs, insulin-like growth factors; IGFBPs, insulin-like growth factor binding proteins.

IGF in cancer

In vitro and *in vivo* studies

In vitro studies have shown that IGF-I exerts mitogenic and anti-apoptotic effects on cancer cell lines (20), including breast (21) and colorectal cancer cells (22). Also *in vivo* studies provide several lines of evidence for a role of the IGF-system in carcinogenesis (23). For example, overexpression of genes encoding either GH or IGF-IR agonists in transgenic mice resulted in both mammary gland epithelial cell hyperplasia and an increased frequency of mammary tumors (24;25). In lit/lit mice, which have a mutation in the GHRH receptor, serum GH and IGF-I levels are reduced to 10% of normal. Transplantation of human MCF-7 breast cancer cells into these mice resulted in a near 100% reduction in tumor growth compared with cancer cells transplanted into normal mice (26). The strongest evidence for a causal association between circulating IGF-I concentrations and cancer risk comes from mice with a liver-specific IGF-I gene deletion (LID mice), as is described in more detail in Chapter 2.

Epidemiological studies

Circulating IGF-I, IGFBP-3 and breast and colorectal cancer risk

One of the first published epidemiological studies investigating the association between cancer risk and the circulating IGF-system was conducted at The Netherlands Cancer Institute by Bruning et al. (27). In this study, an increased breast cancer risk was observed for women who had a relatively high molar IGF-I / IGFBP-3 ratio, which was used as an indicator of IGF-I bioavailability. Since then, many reports have followed, also with respect to other cancers, including colorectal cancer.

In Chapter 2, the prospective cohort studies and case-control studies on circulating IGF-I and cancer published up to 2004 have been summarized, including breast (28-35) and colorectal cancer (36-41). In the last few years, some more reports have appeared with respect to breast (42-48) and colorectal cancer risk (49;50). The literature to date reveals that the association between serum concentrations of IGF-I and premenopausal breast cancer risk has become attenuated with passing years of publication, due to complex reasons including differences in study design, lack of standardisation between assays, and variation in IGFBP-3 proteolytic activity (51). However, the association remains positive and significant (cumulative effect = 1.69, 95%CI 1.17-2.45) (51). This association is even stronger for premenopausal women before age 50 (2.13, 95%CI 1.25-3.64) (51). Although a meta-analysis including studies published up to the end of 2002 revealed a significantly positive association between premenopausal breast cancer and serum IGFBP-3 concentrations (52), a recent meta-analysis including studies published up to the end of 2006 showed a null association (1.11, 95%CI 0.68-1.82), due to statistical heterogeneity among studies (51). Postmenopausal breast cancer risk is not associated with circulating IGF-I and IGFBP-3 concentrations. With respect to colorectal cancer, overall a positive association exists for serum IGF-I concentrations, whereas also here, serum IGFBP-3 concentrations are not associated with cancer risk (53) (**Appendices 1 – 3**).

Circulating IGF-II, IGFBP-1, and IGFBP-2 and breast and colorectal cancer risk

In addition to IGF-I and IGFBP-3, few epidemiological studies have focussed on other circulating IGF-system components with respect to breast and colorectal cancer risk, in particular IGF-II, IGFBP-1, and IGFBP-2. The results are summarized in **Appendices 4 and 5**.

Two cohort studies did not show any associations of IGF-II concentrations with breast cancer risk (42;54). Individuals with Dukes stage A and B colorectal carcinomas were shown to have increased serum IGF-II as well as IGFBP-2 concentrations, as compared to control subjects with a normal colonoscopy (55). Subsequently, in three out of four cohort studies relatively high serum IGF-II concentrations were suggested to be associated with increased colorectal cancer risk (36;39;53;56), and a significantly positive association was found for the three studies combined (53).

Circulating IGFBP-1 and -2 concentrations were not associated with breast cancer risk (30-32;44;46;54), although one study showed a significantly inverse association with postmenopausal breast cancer risk for IGFBP-2 (31). Colorectal cancer risk was significantly inversely associated with IGFBP-1 in two (38;49) out of six cohort studies (38;40;49;50;57;58) and IGFBP-2 in one (38) out of three cohort studies (38;40;58), whereas in the other studies not statistically significant associations were found. Circulating IGFBP-1 and, to a lesser extent, IGFBP-2 concentrations are known to be responsive to insulin (59). However, only three of the studies with respect to breast cancer (32;44;46) and one study with respect to colorectal cancer risk (49) were adequately controlled for fasting status, the latter showing an inverse association for IGFBP-1 (49). The results of the other studies are therefore difficult to interpret. The epidemiological evidence on the association between circulating IGF-system components and breast and colorectal cancer risk is summarized in **Table 1.2**.

Table 1.2. Summary of the epidemiological evidence on the association between circulating IGF-system components and breast and colorectal cancer risk

	IGF-I	IGFBP-3	IGF-II	IGFBP-1	IGFBP-2
Breast cancer					
Premenopausal	Positive	No	-[a]	No	No
Postmenopausal	No	No	-	No	-
Colorectal cancer	Positive	No	Positive	Possible	-

[a] insufficient evidence

Tissue studies

Several studies have investigated mRNA and protein expression of IGF-system components in breast and colorectal tissues. Studies using immunohistochemistry or IGF binding assays showed overexpression of the IGF-IR in breast tumor versus normal breast tissues (60). However, studies investigating IGF-IR mRNA expression using quantitative PCR generally did not confirm this (61-64). Additionally, these studies showed lower levels of IGF-I mRNA expression in tumor tissues compared with adjacent non-cancerous tissue, suggesting a

paracrine role for IGF-I (62-64).
In colorectal tumors, IGF-IR protein was also found to be generally overexpressed (60) and gradually increased during colorectal cancer progression (65;66). Results from quantitative PCR studies were inconsistent, with some studies showing an increase in IGF-IR mRNA expression in colorectal tumor tissue versus normal colorectal tissue (67;68) and some studies not showing any differences (69;70). IGF-II mRNA and protein were also frequently overexpressed in colorectal tumors (68;71-73). The results of these studies in colorectal tissues (65;67-93) are summarized in **Appendices 6 and 7**.
Studies investigating mRNA and protein expression of IGF-system components have used different techniques. Therefore, direct comparisons between studies are difficult, and results thus far are inconsistent and inconclusive (63).

Relation between circulating and local IGF levels

Increased circulating concentrations of IGF-I were found to be associated with breast and colorectal cancer risk, and in breast and colorectal (tumor) tissues IGF-system components are widely expressed. Thus far, it is unknown whether circulating IGF-I and -II proteins are causally related to tumor growth in humans through IGF-IR binding and activation. Alternatively, circulating IGF-I concentrations may influence local tissue expression of IGF-system components (e.g. upregulation of IGF-I, IGF-II, or IGF-IR), or they may be reflective of tissue IGF-system component bioactivity. Results from animal studies concerning this topic are scarce and variable (94). To our knowledge, the relation between endocrine levels of IGF-I and tissue IGF mRNA expression has not been previously investigated in humans.

Determinants of the IGF-system

Circulating concentrations of IGF-system components are determined by both genetic factors (polymorphisms, imprinting) as well as dietary and lifestyle factors (e.g. physical activity), which are extensively reviewed in Chapter 2. Since relatively high circulating IGF-I concentrations are associated with increased premenopausal breast cancer and colorectal cancer risk, modulation of the IGF-system by dietary factors may provide means to reduce this risk. Several dietary factors, such as severe energy and protein restriction, are known to influence the IGF-system but are difficult to implement as a cancer prevention strategy. Some *in vitro* and *in vivo* studies have shown that other dietary components, such as polyphenols (curcumin, green / black tea polyphenols) and flavonoids (apigenin present in fruits and vegetables, resveratrol present in plants including grapes, silibinin present in milk thistle), may also affect the IGF-system (95). However, more detailed information about their safety is needed before their effects on the IGF-system can be investigated in humans (95). The most feasible dietary factors in humans, which are safe and have supportive evidence of an association with the IGF-system, are lycopene and isoflavones. These dietary factors are the subject of this thesis and their characteristics, their relation with cancer, and their relation with the IGF-system are

described in more detail in the next paragraphs.

Lycopene and isoflavones

Lycopene

Lycopene is a member of the hydrocarbon family of carotenoids and accounts for about 50% of the carotenoids found in human blood, thereby being the most predominant carotenoid. It is a natural fat-soluble pigment, and the most potent singlet oxygen quencher and free radical scavenger among all natural carotenoids (96).

Food sources and dietary intake

For most populations in Europe and North America, tomatoes or tomato products are the most important food sources of lycopene. However, lycopene is also present in watermelon, pink grapefruit, guava, papaya, and apricots (97). The major foods contributing to lycopene intake in The Netherlands are tomato soup (29%), tomatoes (16%), and pizza (16%) (98). On average, Dutch men and women consume about 16 grams of tomatoes and tomato products per day. This is the lowest consumption among nine European countries investigated (99). Median dietary lycopene intake in the Netherlands is below 5 mg/day (98;100;101). In line with this, plasma lycopene concentrations are also among the lowest in Europe (e.g. 0.50 $\pm$ 0.30 μmol/L in the Netherlands vs. 1.31 $\pm$ 0.46 μmol/L in southern Italy) (99).

Metabolism and bioavailability

After ingestion, lycopene is incorporated into dietary lipid micelles and absorbed into the intestinal mucosal lining via passive diffusion. These lipid micelles are then incorporated into chylomicrons and released into the lymphatic system for transport to the liver. Subsequently, lycopene is transported by the (very) low-density lipoproteins (VLDL and LDL) into the plasma, and distributed to the different organs where it accumulates in tissues. Lycopene is commonly located within cell membranes and other lipoprotein components, because it is extremely hydrophobic. Lycopene concentrations vary greatly among tissues, suggesting unique biological effects on some tissues, but not on others. The highest concentrations are found in the testes, adrenal glands, liver, and prostate. Lycopene is also present in other tissues, e.g. breast and colon (96;102).

About 10-30% of lycopene is absorbed and the remainder is being excreted. The bioavailability of lycopene is influenced by many biological and lifestyle factors, including age, gender, hormonal status, body mass and composition, blood lipid levels, smoking and alcohol (102). Furthermore, food-related factors can enhance lycopene bioavailability, such as processing (releasing lycopene from its food matrix), the presence of dietary lipids or other carotenoids in the food, and heat-induced isomerization (102). Although about 90% of lycopene in dietary sources is present in the linear, *all trans* confirmation, more than 50% in human blood and tissues is in the *cis* confirmation (9-, 13-, and 15-cis) (103).

This suggests that the *all trans* isomer is changed to *cis* isomers in the acid gastric milieu or that the *cis* isomers are preferentially absorbed (96).
Estimates of lycopene consumption do not necessarily provide an accurate measure of biological exposure, although lycopene concentrations in blood and tissues reflect dietary lycopene intake. The correlation between blood lycopene concentrations and estimated lycopene intake is in the range of 0.21 to 0.47 (104).

Role in breast and colorectal cancer
In 1999, Giovannucci (105) reviewed the epidemiological studies on intake of lycopene (or tomato products as its major source) and cancer risk. The majority of the 72 studies identified reported inverse associations between tomato intake or blood lycopene levels and cancer risk. This decrease in cancer risk was most consistent for prostate, lung, and stomach cancer, but also suggestive for breast and colorectal cancer. The initial strongest significantly inverse associations of tomato and lycopene intake with risk of prostate cancer in observational as well as experimental studies (106) were not confirmed by recent epidemiological studies (107). Currently, a few clinical trials with respect to lycopene supplementation and intermediate markers for prostate cancer are ongoing.
Thus far, also results from case-control and cohort studies examining the association between dietary and blood levels of lycopene and breast cancer risk have remained inconsistent (108). Furthermore, colorectal cancer risk was shown not to be affected by habitual dietary intake of lycopene (mean intake range 0.6 – 10.9 mg/day) in a recent pooled analysis of 11 large cohort studies (109). The association between serum lycopene concentrations and colorectal cancer risk has only been investigated in one recent cohort study, and no association was found for either men or women (110). To our knowledge, the effect of tomato / lycopene supplementation on breast or colorectal risk (markers) has not been investigated.
On the other hand, *in vitro* and *in vivo* studies have shown that lycopene may decrease risk of breast and colorectal cancer. Lycopene has been found to reduce cell proliferation in human mammary cancer cells (111-113), whereas its effect on colorectal cancer cells has not been investigated. In several studies in mice and rats, lycopene supplementation reduced cancer risk, including risk of breast and colorectal cancer, as reviewed by Cohen in 2002 (114). For colorectal cancer, this was confirmed by more recent studies (115-117).
Thus, although *in vitro* and *in vivo* studies do suggest that lycopene decreases risk of breast and colorectal cancer, epidemiological evidence is inconsistent, which may be partly due to the relatively low dietary intake and low correlations between dietary intake and blood lycopene levels. Lycopene may inhibit carcinogenesis due to its antioxidant properties, but also by other mechanisms. These involve improvement of gap-junctional communication, upregulation of detoxification systems, delay of cell cycle progression, modulation of signal transduction pathways, and reduction of cell proliferation (96). Furthermore, lycopene may affect the IGF-system.

Role in IGF-system

Many *in vitro* studies have shown that lycopene can induce cell cycle arrest (97), and several studies found that this effect may (in part) reflect an effect on the IGF system.

In 1995, Levy et al. (111) showed that lycopene inhibits basal cell proliferation in human endometrial, lung, and mammary cancer cells. Additionally, IGF-I-stimulated growth in endometrial cancer cells was also suppressed by lycopene. Subsequent *in vitro* studies found that lycopene reduced IGF-IR expression and/or IGF-IR signaling in human mammary, hepatoma, and prostate cancer cells (112;118-120). These effects were associated with increased concentrations of (membrane-associated) IGFBPs (112;119;120).

An *in vivo* study showed that both low and high dose lycopene supplementation for 9 weeks (equivalent to 15 and 60 mg/day in humans, respectively) increased plasma IGFBP-3 concentrations and decreased lung cancer development in ferrets, whereas plasma IGF-I concentrations were not altered (121). Furthermore, lycopene decreased IGF-I mRNA expression in normal (122) and tumor (123) rat prostatic tissue.

Thus, *in vitro* and *in vivo* evidence suggests that lycopene may affect the IGF-system, possibly through effects on IGFBPs. Also the initially strong inverse association between lycopene and prostate cancer risk has been regulary suggested to be mediated by effects on the IGF-system. However, epidemiological evidence (101;124-128), and in particular evidence from randomised controlled trials (129-131), on the effects of lycopene on the IGF-system in humans is limited and conflicting, and studies addressing this issue are needed.

Isoflavones

Phytoestrogens are polyphenolic plant compounds that have structural and functional similarities to estrogens. They can be classified into three subtypes; isoflavones, lignans, and coumestans (132).

Food sources and dietary intake

The largest quantities of isoflavones are found in soybeans, although many varieties of legumes, vegetables, and grains also contain small amounts. Isoflavone content of these foods depends on variety, geographic location, soil type, year and environmental conditions of growth, and type of processing (133). Examples of soy-containing foods are tofu, tempeh, soymilk, and miso. Over-the-counter food supplements with soy or red clover extracts are an increasing source of isoflavones, especially for women using these as a ‘natural’ substitution for hormone replacement therapy to diminish menopausal complaints. Most dietary sources of isoflavones contain a mixture of derivatives based on three isoflavone aglycones called genistein, daidzein, and glycitein. In soybeans, isoflavones are mainly present in the glucoside form (genistin, daidzin, and glycitin). In red clover, the aglycones genistein, daidzein, biochanin A and formononetin are found. Aglycones are more readily bioavailable and more readily absorbed than glucosides (133). In Asian countries, mean or median intakes of (glucoside) isoflavones range 18 to 63

mg/day, and a substantial proportion of the population consumes at least 100 mg (glucoside) isoflavones per day on a regular basis (133). In Western countries, average daily intakes are generally less than 2 mg isoflavones (133). In the Netherlands, the adult average daily intake is about 1 mg/day (134;135). Among nine European countries investigated, the highest blood concentrations of isoflavones were found in subjects from the Netherlands and the UK (2-6 μg/L ~ 7-24 nmol/L) (136). Although plasma concentrations of isoflavones in Europe were low compared with Asian populations (250-500 nmol/L) (137;138), they varied substantially among subjects from the different countries (8-fold for genistein to 13-fold for daidzein) (136).

Metabolism and bioavailability

After ingestion, acetyl and malonyl glucoside derivatives are metabolized to genistin and daidzin. These are hydrolyzed in the large intestine by bacteria, whereby their sugar moiety is reduced and their respective aglycones, genistein and daidzein, are produced (**Figure 1.3**). Biochanin A and formononetin are also largely metabolized to genistein and daidzein, respectively (139) (Figure 1.3). Following absorption of the aglycones, these compounds are readily conjugated in the liver with glucuronic acid and/or sulfate. They circulate enterohepatically, and are potentially metabolized and reabsorbed in the intestine. They are excreted predominantly in the urine. Isoflavones that are not absorbed are excreted in the unconjugated form in the faeces. Daidzein may be further metabolized by resident microflora in the gastrointestinal tract to equol and O-desmethylangolensin (*O*-DMA) (133) (Figure 1.3). The absorption of isoflavones and the corresponding isoflavone plasma levels were found to be similar after red clover and soy consumption (139;140). However, the bioavailability of isoflavones may be influenced by the type of food matrix or the form in which they are ingested (141). The correlation between blood genistein and daidzein concentrations and estimated genistein and daidzein intake is low for low soy-consuming populations (range of 0.25 to 0.32), whereas it is higher in populations with higher soy intakes and greater variations in intake (142).

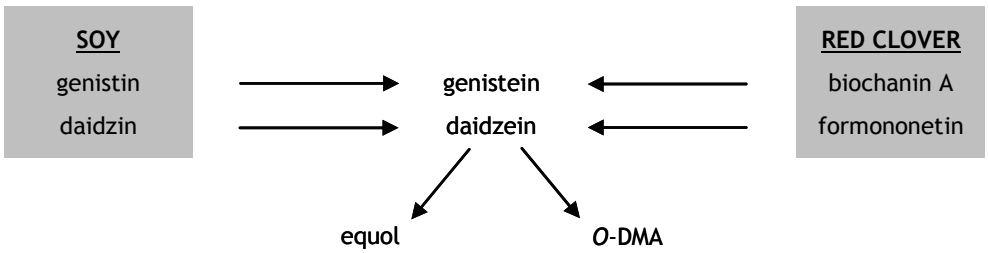

Figure 1.3. Metabolism of isoflavones from soy and red clover (*O*-DMA is O-desmethylangolensin).

Role in breast and colorectal cancer

Breast cancer incidence and to a lesser extent colorectal cancer incidence is markedly lower in many Asian countries compared with Western countries. Furthermore, incidence rates rapidly increase among Asian people following emigration to the United States (143;144). This may be attributed to the reduced ingestion of soy products.

Two recent meta-analyses of epidemiological studies concluded that soy intake may be associated with a small decrease in breast cancer risk (145;146). In experimental studies in humans, no consistent effects of isoflavones on indicators of cell proliferation in normal human breast tissue were found, although isoflavones may increase proliferation in existing breast cancer (147). The relatively few epidemiological studies with respect to colorectal cancer conducted thus far do not show any conclusive trend, partly due to weak methodology (132).

In vitro and *in vivo* studies have shown that low physiological doses ($\leq$10 μM) of isoflavones stimulate breast cancer growth, whereas higher pharmacological doses inhibit tumor growth, possibly due to toxic effects (147). With respect to colorectal cancer risk, *in vitro* and *in vivo* studies are somewhat supportive of a protective role for physiological concentrations of isoflavones, as recently reviewed (132).

Thus, although some epidemiological and experimental studies suggest that isoflavones may decrease breast and colorectal cancer risk, evidence thus far remains inconsistent. Isoflavones may affect cancer risk by influencing estrogen metabolism, inducing cell cycle arrest, antioxidative properties, influencing intracellular signaling, and affecting the IGF-system.

Role in IGF-system

Oral estrogen replacement therapy (148;149) and selective estrogen modulators (150-152) were found to reduce serum IGF-I concentrations. Isoflavones structurally resemble estrogens and possess weak estrogenic activity, and may therefore also decrease circulating IGF-I concentrations.

In vitro studies showed that genistein inhibited IGF-IR signaling in human colon (153) and rat prostate cancer cells (154) in pharmacological doses, whereas physiological doses of genistein had the opposite effect in human breast cancer cells (155). However, physiological dietary concentrations of soy protein or genistein in male mice and rats resulted in a decrease in serum IGF-I (156;157) and IGF-I (158) and IGF-IR content (159) in prostate tumor tissue, whereas in female mice and rats no IGF-effects were found (157;160;161).

Cross-sectional studies in humans did not show a clear association between soy intake and serum IGF-I (162-165). Randomized controlled trials in humans have mainly investigated the effect of soy foods and soy protein isolates containing isoflavones compared with control foods, soy protein isolates or milk protein isolates not containing isoflavones (166-177). However, it is likely that the increase in serum IGF-I levels observed in the majority of studies is mainly due to increased protein intake, which may mask a potential IGF-

lowering effect of isoflavones alone. Therefore, subsequent randomized controlled trials investigating the effect of isolated isoflavones on circulating levels of the IGF-system are warranted.

Rationale and outline of thesis

As has been described in the previous paragraphs, the IGF-system has a potential role in breast and colorectal carcinogenesis. *In vitro* and *in vivo* studies and epidemiological studies have shown that lycopene and isoflavones may affect the IGF-system, and may thereby decrease breast and colorectal cancer risk. However, only few randomized placebo-controlled studies evaluating the effect of lycopene supplementation on circulating IGF-system components have been conducted in humans, and studies were of limited size. Randomized studies investigating the effects of isoflavone supplementation on circulating IGF-system components have mainly used soy foods or soy protein isolates, and potential differential effects of isoflavones apart from soy protein could not be evaluated.

The role of locally expressed IGF-system components in breast carcinogenesis is unclear, and data with regard to mRNA expression of IGF-system components in breast tumor and normal breast tissue are limited and inconsistent. It is also unknown whether this expression differs for women with a family history of breast cancer compared with women without a family history of breast cancer. Furthermore, it is unknown whether circulating IGF-system components are related to tissue mRNA and protein expression of IGF-system components. The research questions of this thesis are depicted in **Figure 1.4**.

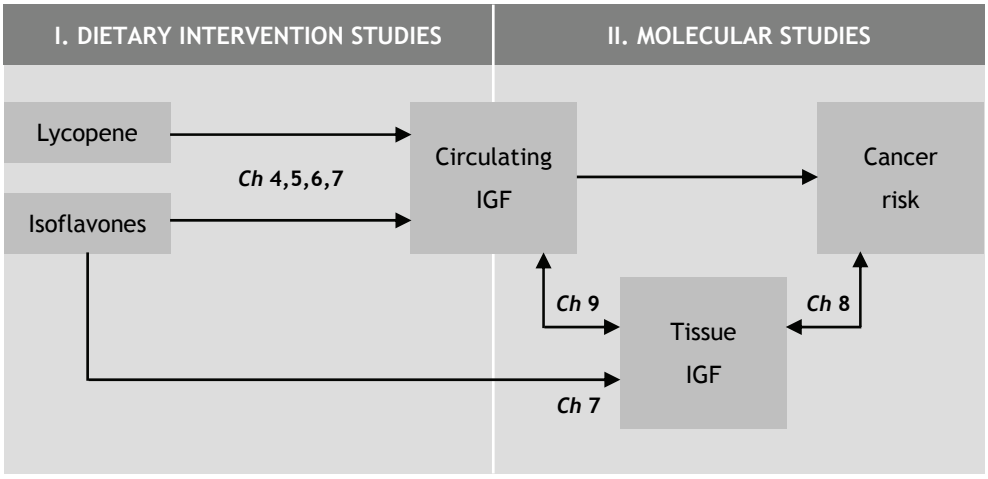

Figure 1.4. Outline of thesis, displaying the research questions and respective chapters in which these are described.

Outline of thesis

In this thesis, first the background about the role of diet in influencing the IGF-system is described (**Chapter 2**). The association between dietary factors, including habitual dietary intake of lycopene and isoflavones and related food products, and circulating IGF-system components in a Dutch female population is evaluated in **Chapter 3**. Randomized placebo-controlled studies evaluating the effect of lycopene and isoflavone supplementation on circulating IGF-system components in populations at increased risk of premenopausal breast cancer and colorectal cancer are described in **Chapters 4 to 7**. In **Chapter 8**, mRNA expression of IGF-system components in normal breast and breast tumor tissue of women with and without a familial breast cancer risk is evaluated. The association between circulating IGF concentrations and mRNA and protein expression of IGF-system components in normal colorectal tissue is described in **Chapter 9**. In **Chapter 10**, the findings of this thesis are integrated and discussed.

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The insulin-like growth factor system in cancer prevention: potential of dietary intervention strategies

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Abstract

The insulin-like growth factor (IGF) system is related to proliferation and tumor growth, and high levels of circulating IGF-I are thought to be a risk factor for several types of cancer. This review summarizes the epidemiologic evidence for an association between circulating IGF-I and cancer risk as well as the experimental evidence for a causal relation between the endocrine IGF system and tumor growth. The potential for dietary intervention to alter the IGF system and thereby cancer risk is supported by several lines of evidence. Postulated mechanisms of action are as follows: (*a*) reduction of levels of circulating IGF-I, which will decrease activation of the IGF-I receptor and subsequent signaling pathways; (*b*) increasing local IGF binding proteins, which may have IGF-dependent effects through obstruction of IGF interaction with local IGF-I receptor as well as IGF-independent effects; and (*c*) interference with estrogens and estrogen receptor action, which may have direct (and possibly synergistic) effects on IGF signaling. An overview is given of the epidemiologic studies on dietary determinants of circulating IGF-I. Examples of dietary factors, such as dairy protein, lycopene, and phytoestrogens, are used to illustrate the potential mode of action of dietary interventions that may act on the IGF system. In conclusion, the IGF system has every potential to serve as an intermediate for cancer (chemo)prevention studies. On the short term, more research initiatives aimed at the effects of specific food components or dietary strategies on the IGF system both in animal models and humans are warranted.

Introduction

The primary role of the human growth hormone-insulin-like growth factor (IGF) axis is the regulation of both prenatal and postnatal growth (1;2). Recent studies point out that the insulin-IGF signaling pathway, which has been conserved in yeast, worms, fruit fly, mice and humans, may be causally linked to aging and longevity (3). Besides regulation of normal growth and aging, the IGF system is also involved in carcinogenesis (**Figure 2.1**; ref. 4).

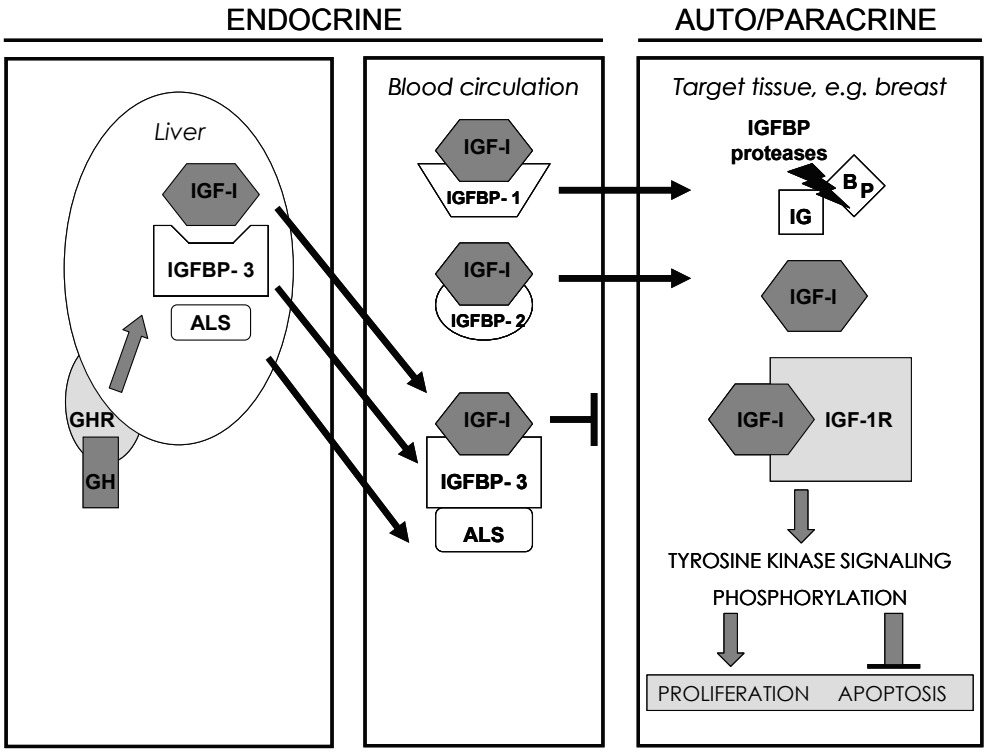

Figure 2.1. The circulating and tissue IGF-system components. Growth hormone stimulates the liver to synthesize IGF-I, IGFBP-3 (its main binding protein in circulation), and acid-labile subunit. About 90% of IGF-I in the circulation is bound to IGFBP-3 and acid-labile subunit in a complex too large to pass the capillary endothelium. Free IGF-I (<1%) and IGF-I bound to IGFBP-1 or IGFBP-2 can be transported out of the bloodstream to specific target tissues. IGFBPs, both in the circulation and in tissues, are degradable by proteases, rendering IGF-I free to interact with IGF-IR. Binding to IGF-IR results in receptor phosphorylation, activation of downstream targets, and stimulation of proliferation and inhibition of apoptosis.

Observational epidemiologic studies suggest that circulating levels of IGF-I and its main binding protein [IGF binding protein-3 (IGFBP-3)] are related to the risk of several epithelial cancers (for a systematic review and meta-regression analysis of the published literature until 2002, see ref. 5). In particular, the results of large prospective studies

consistently show higher risk of breast and prostate cancer, and possibly colon and lung cancer, in individuals with relatively high levels of IGF-I. Variation in circulating IGF-I levels is thought to be due to both genetic effects and environmental or lifestyle factors. Furthermore, the IGF-system is known to interact with insulin (as reviewed in ref. 6) and estrogens at various levels. Animal model systems as well as analyses of human tissue show that the expression of several components of the IGF system may be deregulated in specific tissues, and that normal IGF signaling is disrupted during one or more stages of the carcinogenic process.

In light of recent findings on the association between the endocrine and paracrine/autocrine IGF systems and carcinogenesis and on the association between lifestyle factors and circulating IGF system components, we will provide the rationale for a role of circulating IGF system components in cancer prevention. Several potentially cancer preventive dietary strategies will be highlighted, including the mechanisms by which they may influence the IGF-system and thereby cancer risk.

Overview of the epidemiology

In recent years, many reports have been published on the association between circulating levels of IGF-I and the risk of several types of cancer. In **Figure 2.2**, we summarize the prospective cohort studies as well as those case-control studies that included at least 100 cancer cases. Most valuable are prospective cohort studies, especially if blood samples are drawn well before cancer diagnosis. A positive association in such a prospective study would be indicative of circulating IGF-I as a risk factor for cancer. It should be noted that due to the retrospective design of case-control studies, any association found in these studies could be caused by the presence of the tumor.

In women, the most consistent positive association with IGF-I has been observed for breast cancer in (young) premenopausal women (i.e., relatively high IGF-I associated with increased cancer risk; refs. 7-13). None of the studies on postmenopausal breast cancer observed a positive association (7-15). The fact that the association is absent in postmenopausal women who have very low estrogen levels suggests that the cancer-promoting effect of IGF-I is enhanced by estrogens. A synergistic effect of IGF-I and sex steroids on breast cancer risk was confirmed in a population-based case-control study by Yu et al. (16). The results on the association with IGFBP-3, the main IGFBP in the circulation, are somewhat inconsistent with four out of six studies suggesting a similar positive association with premenopausal breast cancer risk (8-13). Prostate cancer, the most frequently occurring hormone-related cancer in men, is also found to be associated with higher circulating levels of IGF-I in the majority of studies (17-24), especially in relatively young men (18;21). A recent update of the prospective cohort study by Chan et al. suggests that the positive association between IGF-I and prostate cancer risk is limited to advanced-stage prostate cancer (25). Shi et al. (26) have published a meta-analysis of 14 epidemiologic studies on prostate cancer risk, including 7 case-control studies with

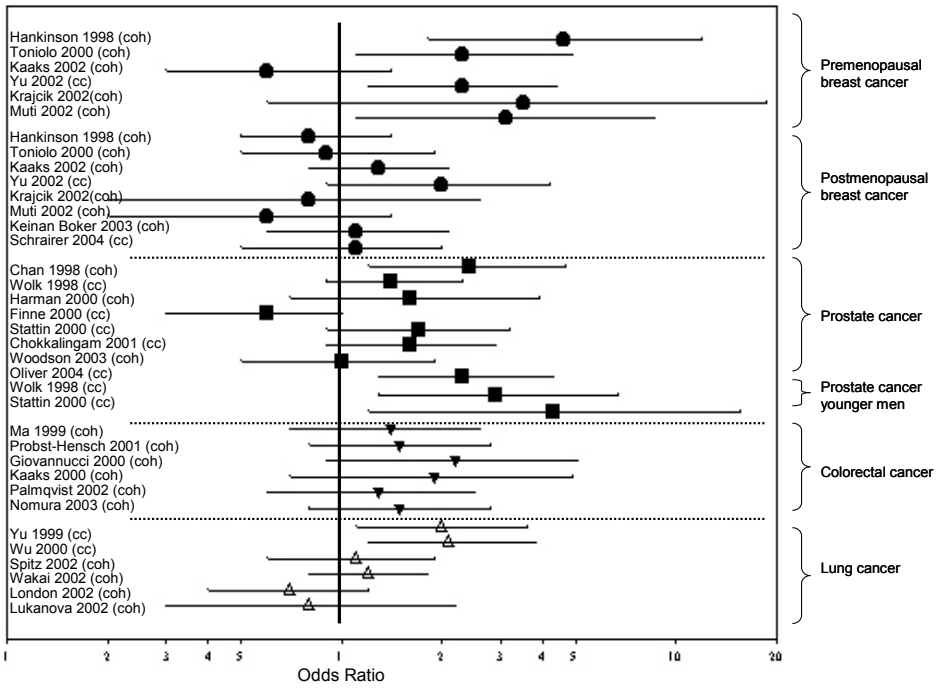

Figure 2.2. ORs (*markers*) and 95% confidence intervals (*horizontal lines*) for cancer risk (cancer death in *Wakai 2002*) in individuals with IGF-I concentration in highest quantile versus lowest quantile. Only prospective cohort (nested case-control) studies (*coh*) are included as well as those case-control studies (*cc*) that included at least 100 cancer cases. Depicted are the ORs that were adjusted for covariates but not for IGFBP-3 (*Giovannucci 2000, Nomura 2003*, and *Wu 2000* were adjusted for IGFBP-3; unadjusted ORs were not reported). Premenopausal breast cancer risk is reported for premenopausal women aged <50 years by *Hankinson 1998* and *Toniolo 2000* and for all women aged <50 years by *Kaaks 2002*.

<100 cases, and reported an increased risk of prostate cancer associated with high concentrations of serum IGF-I [odds ratio (OR), 1.5; 95% confidence interval, 1.3-1.7], as well as IGFBP-3 (OR, 1.2; 95% confidence interval, 1.0-1.5). All six cohort studies (27-32) on the association between colorectal cancer risk and the circulating IGF system found a positive association (i.e., OR, 1.5), although only two were statistically significant and only after adjustment for IGFBP-3 (27) or when analyses were limited to colon cancer (31). Interestingly, two studies reported on the association with IGF-II and both found a positive association, one of which was statistically significant (28;33). The results with respect to IGFBP-3 are inconsistent: two studies report a significant inverse association (i.e., protective effect; refs. 27, 29) and two studies report a significant positive association (28;30). Lung cancer risk has also been studied in relation to serum IGF-I concentrations, but again results are inconsistent, especially those from the prospective cohort studies (34-39). Bladder cancer risk has also been significantly associated with high IGF-I levels (40). Two studies have investigated the association between endometrial

cancer and circulating IGF-I levels and found no significant association (41;42). Overall, a 2-fold increased risk of prostate cancer, premenopausal breast cancer, and possibly colon cancer can be expected in individuals with serum IGF-I levels above the 75th percentile.
Besides the evidence for an association with cancer risk, several studies have been published that show a similar association of serum IGF-I levels with mammographic breast density (43), and precursor lesions such as ductal carcinoma in situ (44), proliferation of colorectal mucosa (45), colorectal adenomas (29;46;47), and benign prostatic hyperplasia (48;49). Obviously, these studies support the evidence for a causal role of increased serum concentrations of IGF-I in one or more stages of the development of several malignancies.
In this respect, it should be noted that relatively high IGF-I levels have also been associated with several possible health benefits, such as decreased risk of cardiovascular disease (50), osteoporosis (51), and less cognitive decline with age (52). Therefore, intervention aimed at reducing IGF-I levels will be especially applicable to individuals at high risk of developing cancer.

Rationale for cancer prevention strategies aimed at the IGF-system

The strongest evidence for a causal association between circulating IGF-I concentrations and cancer risk comes from animal models. In LID-mice, the *igf1* gene is deleted exclusively in the liver, leading to dramatically reduced serum levels of IGF-I (75% decrease) but normal growth and development (53). These mice have delayed onset of chemically and genetically induced mammary tumors (54). When receiving colon tumor transplants, LID-mice develop less colon tumors and liver metastases than wild-type mice (55). A less dramatic reduction in serum IGF-I (25% decrease) due to energy restriction also decreased malignant transformation in a mouse model, whereas restoration of IGF-I concentrations by recombinant IGF-I stimulated cell proliferation (56). Besides energy restriction, several other dietary or pharmaceutical interventions in animal models have been shown to result in changes in serum levels of IGF-system components, thereby altering tumor growth, apoptosis, and other processes related to cancer risk. Several examples of such studies will be described in the next paragraph.
Clearly, the number of experimental animal studies on the IGF system in cancer prevention is increasing. Although the results of these studies cannot be easily generalized to humans, it will help to elucidate the underlying mechanisms. Overall, the current evidence from studies in model systems points to the IGF system as a feasible cancer prevention target. If variation in circulating levels of (free) IGF-I is also causally associated with cancer risk in humans, even marginal alterations in the serum concentration of IGF-system components may provide an interesting contribution to cancer prevention. To further investigate the potential of diet and the IGF system in cancer prevention, two questions need to be addressed. First, intervention studies are needed to investigate whether the concentration of (free) circulating IGF-I in humans is

modifiable by exogenous factors, such as lifestyle changes or pharmaceutical / dietary intervention. Second, the effect of marginal changes in serum IGF-I concentrations in humans needs to be studied in relation to the risk of several types of cancer as well as their precursor lesions.

With respect to the first question, it is not yet clear which factors determine the circulating levels of IGF-system components in humans. Genetic variation is estimated to account for 40% to 60% of the total variation in circulating levels of IGF-I and IGFBPs (57;58). Therefore, ~50% of the variation should, in principle, be modifiable by exogenous factors (e.g., dietary habits or other lifestyle factors). Manipulation of the insulin pathway may well have beneficial effects on the IGF system (6). Many studies have investigated the association between physical activity and the IGF system, although the results remain inconsistent (59). It is a well-known phenomenon that, in cases of chronic or acute energy restriction, serum levels of IGF-I are strongly reduced (60;61). However, the evidence with regard to most dietary factors in humans is limited to observational epidemiologic studies (62-74). In **Table 2.1**, we summarize the evidence from studies on animal models and from cross-sectional and experimental studies in humans. The evidence is inconclusive on all dietary factors, although it suggests that energy and alcohol intake within the normal range are not associated with IGF-I. This summary table does suggest the following: *(a)* intake of animal protein and dairy products may possibly be positively associated, and intake of some minerals is probably positively associated, with IGF-I; *(b)* consumption of tomato products and/or intake of lycopene may be inversely associated with IGF-I (or the ratio of IGF-I/IGFBP-3) and positively with IGFBP-3; however, the evidence is insufficient; and *(c)* with respect to consumption of soy and/or intake of isoflavones, the evidence remains insufficient (i.e., they may have opposing effects that could not be disentangled in studies conducted thus far).

Besides these studies of lifestyle factors, some promising pharmaceutical interventions, such as selective estrogen receptor (ER) modulators (e.g., tamoxifen), synthetic retinoic acids (e.g., fenretinide), and cyclo-oxygenase 2 inhibitors (e.g., celecoxib), have also been associated with the IGF system in model systems and are currently being evaluated in clinical trials. Although the IGF system is not the main end point in these trials, they can provide additional insight into the mechanisms involved in humans. Obviously, such pharmaceutical interventions may prove to be only suitable and acceptable for individuals at very high risk, or those already diagnosed with cancer, and may not be desirable for primary prevention in healthy individuals.

Understanding dietary mechanisms

The relationship between concentrations of IGF-I and its binding proteins in the circulation, and tissue expression/content of IGF system components, as well as the mechanisms through which both may be related to cancer risk in humans, have not been clarified. Certainly, circulating levels of IGF-I and its binding proteins have endocrine

Table 2.1. Summary of the evidence for associations between dietary factors and serum IGF-I

Nutrient/ food group	Studies using animal models[a]	Cross-sectional studies in humans[b]	Experimental studies in humans[a]	Conclusion
Energy	Severe energy restriction results in 25% decreased serum IGF-I (review; ref. 75)	No association (8x; refs. 68, 63, **69**, 71, 66, 72, **73**, 74) Positive association (2x; refs. **67**, 70)	Serum IGF-I is markedly lowered by energy deprivation (review; ref. 60)	Probable; positive association with extremes of energy intake No association within normal range of energy intake
Protein (total or animal)	Severe protein restriction results in 50% decreased serum IGF-I (review; ref. 61)	No association with total and/or animal protein (6x; refs. 64, 66, 71, 72, **73**, 74) Positive association with total or animal protein (5x; refs. 63, **67**, 68, **69**, 70)	Serum IGF-I is markedly lowered by protein deprivation (review; ref. 60)	Possible; positive association with intake of animal protein
Alcohol	Chronic alcohol feeding may increase or decrease serum IGF-I	No association with IGF-I (5x; refs. 64, **67**, 68, 72, 74) Minor inverse association (66) Positive association (in men only; ref. **62**)	Decline in serum IGF-I after drinking alcohol (1 d only)	No association within normal range of alcohol consumption
Minerals (total or zinc)	Mineral (e.g., zinc, copper, magnesium) deficiency is associated with low serum IGF-I levels	No association with zinc (68) Positive association with zinc (63, **67**) Positive association with combined intake of 5 minerals (including zinc; ref. **69**)	Zinc supplementation: increased serum IGF-I levels in specific populations (anemic, non-insulin-dependent diabetes mellitus)	Probable; positive association with zinc or a combination of minerals (including supplements)
Dairy products	Only studies relating to IGF-I content in milk (review; ref. 79)	No association with total dairy (64, 66, 68, 71; one positive association with milk only; ref. 71) Positive association with total dairy (**67**) Positive association with milk (65, **69**)	Serum IGF-I increased 10% with three servings of dairy daily (77)	Possible; positive association with consumption of dairy/ milk

(Table 1 continued)

Nutrient/ food group	Studies using animal models[a]	Cross-sectional studies in humans[b]	Experimental studies in humans[a]	Conclusion
Soy or isoflavones	Decrease in serum IGF-I in animals on soy protein/ phyto-estrogen diet (93, 94)	Asian populations: no association with soy or isoflavones (72); positive association (in men only; ref. **73**) European population (including vegans/ vegetarians): no association with soy protein (borderline positive with soy milk; ref. 68) European population (low intake): no association with soy or isoflavones (74)	Soy protein vs. milk protein: stronger increase in IGF-I in soy protein group (99,100) Soy protein with vs. without isoflavones: trend towards lower IGF-I with isoflavones (postmenopausal women only; ref. 98) or no difference between groups (101)	Insufficient; association could be positive or inverse, depending on dose and population Effects of soy protein and isoflavones/ phytoestrogens need to be disentangled (possibly opposite effects)
Tomatoes or lycopene	Increased plasma IGFBP-3 concentration in ferrets receiving lycopene ((75))	Tomato-products; No association (64, 71, 74; 1x inverse with IGF-I/ IGFBP-3 ratio; ref. 71); inverse association (66) Lycopene intake; No association (positive with IGFBP-3; ref. **67**)	Lycopene intervention: IGF-I decreased in both intervention and control groups (85)	Insufficient; inverse association with IGF (or IGF/IGFBP-3 ratio), positive association with IGFBP-3

NOTE: "Probable": The association (or lack of) is consistently shown in cross-sectional studies and confirmed by experimental studies in animals and humans. "Possible": Association in some but not all cross-sectional studies, experimental studies in animals consistently show the association; however, no or only one experimental study in humans is available. "Insufficient": Only very few studies have been conducted and these are not entirely consistent; [a] References are only included if a review paper is available or if literature is limited to one or two studies; [b] All published cross-sectional studies (n = 13) are included; references of studies including >500 individuals are printed in bold.

effects on specific target tissues. Additionally, they may be markers of overall tissue expression of these IGF system components, thus reflecting paracrine/autocrine IGF effects. Alternatively, regulation of the circulating and tissue IGF systems may be (partly) independent.

How might dietary factors be able to influence circulating IGF system components and/or tissue IGF-systems in humans? Many hypothetical mechanisms can be proposed (**Figure 2.3**). First, as 80% of circulating IGF-I are synthesized in the liver, the most obvious hypothesis would be that exogenous factors directly influence hepatic (or overall tissue) IGF-I expression, synthesis, and secretion, resulting in altered serum levels. Second, the diet may interfere through alteration of IGFBP levels, thereby influencing the ability of IGFs to bind to the IGF-I receptor (IGF-IR) and its resulting signaling cascade. Third,

dietary factors may indirectly affect the IGF system through its interaction with estrogen action and/or ER signaling. Each of the above-postulated mechanisms are described in the sections below, including examples of potential dietary determinants and the available evidence from *in vitro* studies, animal models, and studies in humans.

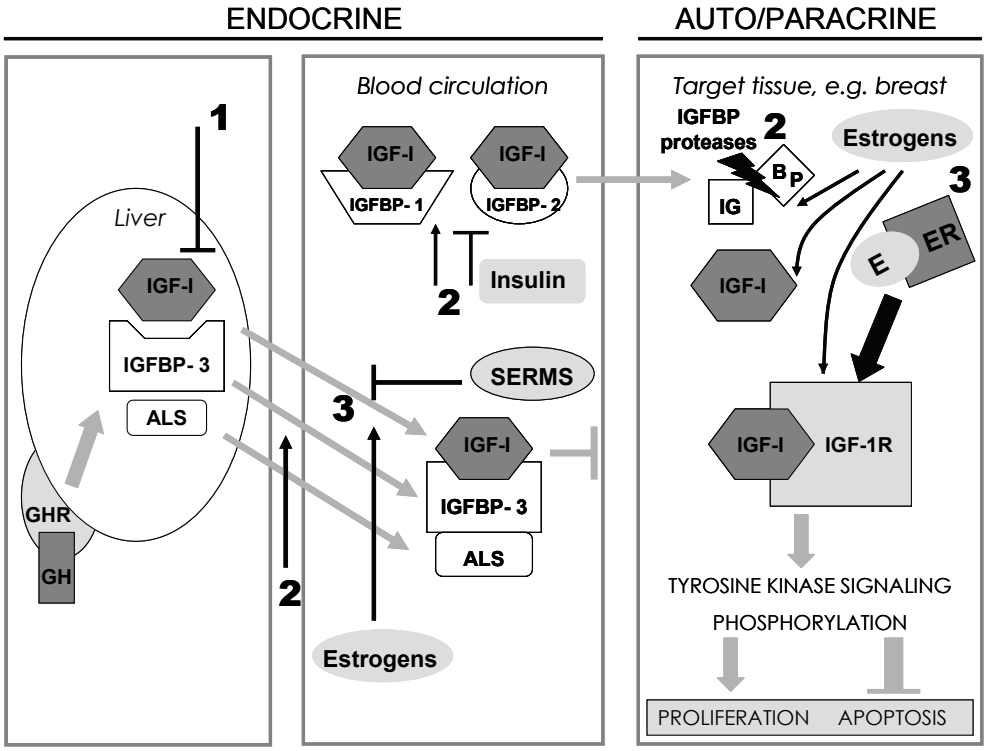

Figure 2.3. Potential mechanisms of direct or indirect effects of exogenous factors on the IGF-system. The first potential mechanism represents effects on the levels of circulating IGF-I. The most obvious pathway through which this may occur is through inhibition of hepatic IGF-I synthesis (*1*). A second mechanism through which IGF signaling might be altered is indirectly through effects on IGFBPs. For instance, synthesis of (hepatic) IGFBP-3 may be stimulated, levels of insulin and other hormones may affect IGFBP-1 and IGFBP-2 levels, and IGFBP degradation may be influenced through effects on proteases (*2*). The third possible mechanism involves estrogens and the ER: they can affect almost every possible component as well as the interaction between components in the IGF system (*3*).

Effects on Circulating IGF-I

Hepatic IGF-I expression and thus circulating IGF-I levels may be influenced by exogenous factors either directly or indirectly. Because growth hormone stimulates the liver to produce a large proportion of circulating IGF-I, indirect inhibition of hepatic IGF-I expression/synthesis may occur through decreased growth hormone receptor binding or post-receptor resistance to growth hormone. Reduced steady-state levels of hepatic mRNA

for IGF-I (due to reduced mRNA synthesis or enhanced degradation) or reduced translation of IGF-I mRNA to protein will reduce serum IGF-I and may result in altered IGF-signaling in specific tissues through changes in the amount of IGF-I available for receptor binding.
The decreased serum IGF-I levels observed after chronic or acute energy restriction (60;61;76) are found to be mainly due to effects on liver IGF-I mRNA levels, although reductions have also been observed in most other tissues studied (60). Dietary administration of corticosterone, which is usually increased during energy restriction, not only resulted in decreased plasma IGF-I concentrations in a rat mammary carcinogenesis model but also in reduced IGF-1R levels in mammary carcinomas, inhibition of several downstream signaling pathways, and reduced incidence of carcinomas (76).
Dairy protein is another example of a dietary factor that is thought to affect serum IGF-I levels in humans possibly through hepatic stimuli. Several cross-sectional studies have found that serum IGF-I concentrations are positively associated with protein intake (63;69;70), specifically animal and soy protein (67;68). In four such studies, consumption of dairy products, a source of animal protein, was positively associated with serum levels of IGF-I (65;67;69;71). In a human intervention study with three servings of dairy products daily, a 10% increase in serum IGF-I was observed (77). From these epidemiologic studies, it cannot be concluded which component in dairy foods might be responsible for this effect and through which mechanism. It may be due to the relatively high level of essential amino acids in dairy protein, resulting in increased hepatic IGF-I synthesis. Studies that tried to disentangle the effects of calcium and dairy protein have been inconsistent (65;67;71). Some investigators suggest that the effects of dairy could be due to bovine IGF-I, which is identical to human IGF-I (78) and may be absorbed in the human resulting in a direct increase in serum IGF-I (79).

IGFBPs and IGF Signaling

In the circulation, 80% to 90% of IGF-I are bound to IGFBP-3 and acid-labile subunit in a complex too large to exit the bloodstream, whereas only <5% of IGF-I are bound to IGFBP-1 or IGFBP-2 in smaller complexes that facilitate transport to other tissues (Figure 1). At the cellular level, formation of complexes between IGF-I and any of its binding proteins prevents IGF-I from binding to the IGF-1R, thus inhibiting IGF signaling. Dietary factors may influence the IGF system by affecting transcription or degradation of IGFBPs (Figure 3). For example, dietary energy restriction not only decreases serum IGF-I concentrations but also decreases serum IGFBP-3 concentrations and increases serum IGFBP-1 and IGFBP-2, potentially resulting in accelerated clearance of IGF-I (i.e., more rapid distribution of IGF-I into tissues; ref. 61). The exact role of the main IGFBPs in serum (IGFBP-3, IGFBP-2, and IGFBP-1, respectively) in the regulation of both endocrine and paracrine/autocrine IGF-I action is largely unknown, making it difficult to interpret the effects of dietary factors on IGFBPs.
One example of a dietary factor that may exert its cancer-preventive actions by influencing IGFBPs is lycopene, a tomato-derived substance. Many experimental studies

have shown that lycopene can induce cell cycle arrest (80). Several studies show that this effect may (in part) reflect an effect on the IGF system. In 1995, Levy et al. (81) showed that lycopene inhibits basal cell proliferation (measured by thymidine incorporation) in endometrial, lung, and mammary cancer cell lines. More specifically, IGF-I-stimulated growth in endometrial cancer cell lines was repressed by lycopene. Karas et al. (82) showed that growth stimulation of MCF7 mammary cancer cells by IGF-I was also reduced by lycopene. Moreover, in this system, lycopene was shown to inhibit activation of the IGF-1R (i.e., reduced tyrosine phosphorylation of insulin receptor substrate 1 and binding capacity of the activator protein 1 transcription complex). This was not due to alterations in the number or affinity of IGF receptors but to increased amounts of membrane-associated IGFBPs. A recent study of lycopene in an experimental animal model (ferrets) reported effects on the endocrine IGF system (i.e., increased serum IGFBP-3 and decreased serum IGF-I/IGFBP-3 ratio) as well as on cancer development (i.e., induction of apoptosis and fewer smoke-induced squamous metaplasia in the lungs; ref. 83).

In 1999, Giovannucci (84) reviewed the epidemiologic studies on intake of lycopene (or tomato products as its major source) and cancer risk. A total of 72 studies were identified, the majority of which reported inverse associations between tomato intake or blood lycopene level and the risk of cancer at a defined anatomic site, most consistently for prostate, lung, and stomach cancer.

Lycopene is a known quencher (the most efficient of all carotenoids) of singlet oxygen and free radicals. Therefore, lycopene is often thought to decrease cancer risk through a reduction in oxidative damage. However, the experimental evidence described in the previous paragraph suggests that alternative pathways, such as the IGF system, may be possible. The number and quality of human studies on the effects of lycopene on the IGF system are insufficient. In one small clinical trial of lycopene supplementation, a reduction in plasma IGF-I and IGFBP-3 levels was observed (85). However, as the serum lycopene concentrations did not significantly increase, the changes to the IGF system may well be due to chance. In cross-sectional epidemiological studies, high consumption of cooked tomatoes was associated with low serum IGF-I levels (66) and high lycopene intake was associated with high IGFBP-3 levels (67).

Altogether, the available evidence thus far suggests that lycopene may alter serum/tissue levels of IGFBPs, thereby indirectly influencing bioactive IGF-I levels and IGF signaling. Future experimental studies both in animal models and in humans should incorporate assessment of IGFBP levels as well as the concentration of free IGF-I in the circulation.

Interaction with Estrogen and ER activity

Besides direct dietary effects on components of the IGF system, there may be indirect influences through estrogen and ER signaling (Figure 3). Estrogen action is strongly related to the IGF system with evidence for cross-talk between the two systems at several levels: Expression of most IGF system components is altered by estradiol (E2), the IGF-1R is directly activated by liganded ER, IGF signaling transcriptionally activates the ER, and

IGF-I and estrogen have synergistic effects on cell cycle signaling cascades and proliferation (86).

Studies on the serum IGF-I reducing effects of tamoxifen provide proof of principle for the action of selective ER modulators on the IGF system (87-90). Phytoestrogens (e.g., isoflavones from soy) are also thought to act like selective ER modulators. Moreover, some epidemiologic observational studies have also shown that the risk of several types of cancer is decreased in populations with high intake of soy products (91;92). The proposition that phytoestrogens may also interfere with the IGF system is mainly derived from these known effects of estrogen and selective ER modulators. Thus far, there is limited direct evidence for an inhibiting effect of phytoestrogens on IGF-I concentrations or IGF system signaling (93). Zhou et al. (94) found a decrease in serum IGF-I and inhibition of growth of transplantable human prostate carcinoma in mice on a soy protein/phytoestrogen diet. Mentor-Marcel et al. (95) found reduced tumor formation in a rat prostate cancer model on an isoflavone-enriched diet, and Lamartiniere et al. (96) described the down-regulation of expression of androgen receptor, ER, progestrone receptor, and IGF-I mRNA in these rats. Flaxseed, another source of phytoestrogens (i.e., lignans), was also observed to down-regulate IGF-I expression in a nude mice model (97).

Four intervention studies in humans have thus far investigated the IGF effects of soy protein with isoflavones in comparison to either milk protein or soy protein without isoflavones (98-101) . The two small parallel studies of soy protein versus milk protein in men and postmenopausal women ($n < 50$) observed increased levels of serum IGF-I in both intervention groups but a significantly stronger increase in the soy protein group (99;100). A small crossover study included both premenopausal and postmenopausal women; in the premenopausal women, a marginal increase in IGF-I in the low isoflavones but not the high isoflavones period was observed (98). In contrast, in postmenopausal women, a trend toward decreased IGF-I levels with increasing isoflavone consumption was observed. In a mixed population ($n = 150$), during a 1-year intervention, no change in IGF-I levels was observed in the high isoflavones group nor in the control group that received soy protein without isoflavones (101). Part of the inconsistency between these studies may be due to chance (i.e., small sample size) or to differences in effect between men and women, and between premenopausal and postmenopausal women, possibly due to interactions with endogenous estrogens. Additionally, opposing effects have been suggested for soy protein (IGF-I increasing) and isoflavones (IGF-I decreasing), which would be difficult to disentangle in studies using soy protein products as a source of isoflavones. Further insight may be provided by studies that do not use soy protein but isoflavone extracts (e.g., from soy or red clover).

Conclusions

Prospective epidemiologic studies have shown that individuals with relatively high serum concentrations of IGF-I are at increased risk of prostate, colon, and premenopausal breast cancer. Abundant experimental evidence is available for an important role of the IGF system in promoting cancer growth. Evidence for a causal association between the circulating IGF system and cancer in humans is still missing, but such evidence from animal models is accumulating rapidly. Additionally, the number of studies that suggest that the circulating IGF system can be altered by dietary interventions, in a direction that may help prevent cancer, is increasing. This includes observational studies in humans that show that certain specific dietary factors may be associated with the concentration of IGF system components in serum, although the results of these studies are not entirely consistent. Experimental studies in animal models show that dietary intervention affects serum IGF system components as well as the occurrence of cancer in these animals.

IGF-related mechanisms by which dietary interventions, such as energy restriction, lycopene supplementation, and phytoestrogen supplementation, may reduce cancer risk are inhibition of circulating IGF-I-induced proliferation and tumor growth, obstruction of IGF signaling in target tissues by increasing local IGFBPs and thus decreasing IGF-1R activity, and interference with estrogen and ER action that affects IGF signaling.

In conclusion, the IGF system has high potential to serve as an intermediate system that can be altered to decrease cancer risk by means of possibly more than one dietary intervention strategy. Additional research is needed. First, dietary intervention studies of relatively short duration should be conducted in relevant human populations to investigate, in a highly controlled manner, the effect of such interventions on serum concentrations of total and free IGF-I and of IGFBP-1, IGFBP-2, and IGFBP-3. Where possible, tissue-specific effects on IGF system components and signaling should also be studied in humans. Experimental studies in model systems can help decide which dietary strategies are most effective. In a later stage, this may accumulate to enough evidence to conduct large, long-term human intervention studies with precursor lesions and eventually cancer as the end points.

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Dietary determinants of circulating insulin-like growth factor (IGF)-I and IGF binding proteins 1, -2 and -3 in women in the Netherlands

3

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Abstract

Objective: Epidemiological studies suggest that individuals with elevated plasma concentrations of insulin-like growth factor (IGF)-I are at increased risk of developing cancer. We assessed whether dietary intake of total energy, protein, alcohol, phytoestrogens and related foods, and tomatoes and lycopene was associated with plasma levels of IGF-I and IGF binding proteins (IGFBPs) in Dutch women.

Methods: A cross-sectional study was conducted in 224 premenopausal and 162 postmenopausal women, aged 49-69, participating in the Prospect-EPIC study in the Netherlands. Diet was assessed using a food frequency questionnaire.

Results: In postmenopausal women, higher alcohol intake was associated with lower plasma IGFBP-1 concentrations (alcohol 1.4 to 20 g/day: 20% decrease in IGFBP-1; $p = 0.04$), and higher intake of plant lignans was associated with higher IGFBP-1 concentrations (plant lignans 0 to 1 mg/day: 59% increase in IGFBP-1; $p = 0.02$). Higher soy intake was associated with higher plasma IGFBP-2 concentrations in premenopausal women (soy 0 to 2.5 g/day: 3% increase in IGFBP-2; $p = 0.04$). No independent associations of dietary factors with IGF-I or IGFBP-3 concentrations were observed. However, in premenopausal women alcohol intake was inversely associated with IGF-I and positively associated with IGFBP-3 after mutual adjustment.

Conclusions: In this study population, with limited variation in dietary intake, total energy, protein, phytoestrogens and lycopene were not associated with IGF-I and IGFBP-3. Alcohol was inversely, and some measures of phytoestrogen intake were positively associated with plasma IGFBP-1 or –2 concentrations. The roles of IGFBP-1 and –2 in relation to IGF-I bioactivity and cancer deserve further investigation.

Introduction

Insulin-like growth factor I (IGF-I) is important in normal growth and development. However, it has also been shown to promote tumor growth (1). Prospective cohort studies have observed that subjects with elevated levels of IGF-I are at increased risk of developing several types of cancer, including prostate, premenopausal breast, and colorectal cancer (2-8). IGF action is determined by the availability of IGF-I to interact with the IGF-I receptor, and is dependent not only on absolute IGF-I concentrations, but also on the relative concentrations of several IGFBPs (9). A high concentration of IGFBP-3, the main IGF binding protein in the circulation, is thought to reduce IGF-I action. However, studies on the association between plasma IGFBP-3 levels and cancer risk remain inconclusive (2;3;5-8). Only a small fraction of IGF-I in the circulation is bound to IGFBP-1 and IGFBP-2, in complexes which allow transport of IGF-I out of the bloodstream, possibly resulting in increased levels of IGF-I in tissues. Ultimately, at the receptor level, binding of IGF-I to any of the IGFBPs inhibits IGF-action. Serum levels of IGF-I as well as the IGFBPs vary substantially between subjects, and are determined by both genetic and environmental factors, e.g. dietary factors (10).

Previous studies investigating the regulation of the IGF-system by diet have mainly focused on total energy and protein intake. Severe energy and protein restriction was found to lower serum IGF-I levels in both animals and humans (11). Results of cross-sectional studies investigating the relation between the normal range of energy and protein intake and serum IGF-I levels are conflicting (12-20), with only few studies showing a significant positive association (21-24). Alcohol consumption can possibly increase IGF-I production by the liver (25), but has been inconsistently related to IGF-I and IGFBP-3 levels in cross-sectional studies (14;15;17;22;26).

Several micronutrients or bioactive compounds in foods may also affect the IGF-system. Estrogen action is strongly interrelated with the IGF-system, and selective estrogen receptor modulators (e.g. tamoxifen, raloxifene) have been shown to lower serum IGF-I levels (27-29), and may elevate some IGFBP levels (27;28). Phytoestrogens are plant substances that structurally resemble endogenous estradiol and are able to bind to its receptor (30). Therefore, we hypothesized that phytoestrogens may also potentially decrease IGF-I concentrations. Three human intervention studies investigated the effect of soy protein, an important source of phytoestrogens, on serum IGF-I, but produced conflicting results (31-33). Additionally, results differed for pre- and postmenopausal women (31), which suggests that the effects of phytoestrogens on the IGF system components may be dependent on endogenous estradiol concentrations. Lycopene is an anti-oxidant mainly found in cooked or processed tomatoes, and has been suggested to interfere with IGF signaling in experimental studies (34-36). Also in cross-sectional studies lycopene (22) or cooked or processed tomatoes (14;18) were associated with IGF-I or IGFBP-3.

The objective of the present cross-sectional study was to assess whether dietary intake of total energy, protein, alcohol, phytoestrogens and related foods, and tomatoes and

lycopene, was associated with plasma levels of IGF-I or with any of its three main binding proteins (IGFBP-1, -2, and -3) in premenopausal and postmenopausal Dutch women.

Materials and Methods

Study population

The study population is a sample of premenopausal and postmenopausal participants of the Prospect cohort which is part of the ongoing European Prospective Investigation into Cancer and Nutrition (EPIC) (37). In the Prospect-EPIC study a total of 17,357 women, living in Utrecht (the Netherlands) and surroundings, were enrolled between 1993 and 1997 and are subsequently followed for the occurrence of cancer. Women were recruited among participants of the nation-wide breast cancer screening program, and 49-69 years of age. The participation rate of the Prospect-EPIC study was approximately 35% (38).

Premenopausal women did not previously undergo bilateral ovariectomy, reported at least 10 menses during the last 12 months, and did not use any hormones for contraception or (peri)menopausal complaints. The total number of women meeting these selection criteria represented 9% (n = 1357) of all women in the cohort. We further excluded women with insulin-dependent diabetes mellitus (n = 7) or a diagnosed malignancy at baseline (n = 66), as these diseases may affect circulating hormone/peptide levels. Additionally, for practical reasons, we had to exclude 12 women because one or more questionnaires were missing, as well as 335 women because no plasma was available for cross-sectional analyses (i.e. all women enrolled before 1995). A resulting total of 937 eligible women was available for further study. As has been described previously (39), we over-sampled 50 women who performed regular physical activities of moderate intensity for at least 30 min/day, and 48 women who reported not performing physical activities. We randomly selected 126 women from the remaining eligible women, resulting in a selection of a total of 224 premenopausal women.

We further included 163 cancer-free postmenopausal women from a previously conducted case-control study on circulating IGF levels and risk of postmenopausal breast cancer (40). They were matched to each breast cancer case at 2:1 ratio (+10%) by age ($\pm$ 1 year), and date of enrollment. These women reported their last menstruation 12 months or more before time of enrollment, and were non-users of hormone-replacement therapy (HRT) and insulin. One woman was excluded because of missing data on food intake. In total, we have selected 224 premenopausal and 162 postmenopausal women as our study population.

Data collection

Baseline information was collected using self-administered questionnaires and a standardized medical examination. The general questionnaire consisted of questions on demographic characteristics, presence of chronic diseases of interest, and potential risk factors for cancer, i.e. reproductive history, family history, drinking of alcohol, smoking

habits, and physical activity. The "total physical activity score" is a summary measure, combining household, occupational and recreational physical activity (41). Missing values for physical activity score were imputed by the mean value of pre- or postmenopausal women (n=27).

The self-administered food frequency questionnaire (FFQ) referred to habitual intake of 178 food items during the preceding year (42;43). The questionnaire contained color photographs of 2 to 4 different-sized portions of 21 food items, which helped assessing the serving size. Subjects indicated their consumption frequency of each food item on a daily/weekly/monthly/yearly scale or as never consumed. An adapted version of the 1993 computerized Dutch food composition table was used to calculate energy and nutrient intake. To calculate lycopene intake, an adapted version of the 2001 computerized Dutch food composition table was used. Phytoestrogen intake was calculated using published laboratory analysis data for the phytoestrogen contents of relevant food items (44;45). Total phytoestrogens is calculated as the sum of total isoflavones (daidzein, genistein, formononetin, biochanin A) and plant lignans (matairesinol, secoisolariciresinol).

The physical examination included measurements of blood pressure and anthropometric indices (i.e. height, weight, waist and hip circumferences), as well as the collection of a 30 ml blood sample. The blood collection protocol included recording of time since last consumption of food or drinks (median: 2 h, range 2 min – 13 h). Hemolysis of the blood samples has been recorded and samples were excluded if any severe hemolysis had occurred. All blood samples were drawn and processed by qualified research assistants and stored in liquid nitrogen (-196 ^{o}C) in 500 μl plastic straws, until transportation on dry-ice to Lyon, France.

IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 assays

Plasma concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were analyzed in the Hormones and Cancer Laboratory at the International Agency for Research on Cancer, Lyon, France, using immunoassays from Diagnostic Systems Laboratories (Webster, Texas, USA). For measurement of IGFBP-2 a competitive radioimmunoassay was used. IGF-I, IGFBP-1, and IGFBP-3 were measured by double-antibody immunoradiometric assays. The IGF-I assay was preceded by an acid-ethanol precipitation procedure to extract IGF-I from its binding proteins. Control samples were included in each batch to calculate inter-assay coefficients of variation (CV). The detection limit varied between 0.01 and 0.80 ng/ml for these assays. In one sample a IGFBP-1 concentration below the detection limit was found, and this value was excluded from statistical analyses. Samples for postmenopausal women were analysed a year after those for premenopausal women. The intra- and inter-assay CVs ranged between 1.1% and 8.5%, and between 4.7% and 17.0%, respectively (39;40).

Data analysis

The following dietary factors were investigated: total energy, protein (total, animal, plant), alcohol, phytoestrogens (total phytoestrogens, total isoflavones, plant lignans) and

related foods (soy, legumes, cereals), tomatoes (raw and processed), and lycopene. Food groups consisted of the following items: soy products (tempeh, tofu, vegetarian burgers); cereals (pasta, rice, other grains, bread, crispbread, rusks, breakfast cereals, crisps and salty biscuits, dough and pastry); processed tomatoes (cooked tomatoes, concentrated tomato paste, tomato sauce). Total, animal, and plant protein were adjusted for total energy intake using the residual analysis method (46). All analyses were conducted for pre- and postmenopausal women separately, because these two groups were selected separately, and blood analyses for both groups were performed with a one year interval. In addition, due to the known interaction between estrogens and IGF, the relation between dietary factors and the IGF-system might be different for pre- and postmenopausal women.

Pearson correlation coefficients were calculated to assess the association between plasma levels of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 and dietary factors. Pre- and postmenopausal women were categorized separately, based on tertiles or relevant categories of dietary factors, and mean concentrations of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were calculated in each category of dietary factors. Tertiles were used for total energy, protein, cereals, and lycopene. For alcohol, soy, legumes, and tomatoes, relevant categories were used: for alcohol <1 glass/week (1.43 g/day), 1 glass/week - <2 glasses/day (20 g/day), and ≥2 glasses/day; for soy 0, >0 – <2.5 (=median intake of soy consumers), and ≥2.5 g/day; for legumes <1 (7 g/day), 1 – <2 (14 g/day), and ≥2 serving sizes/week; for raw tomatoes <0.5 (5 g/day), 0.5 – <1 (10 g/day), and ≥1 tomatoes/week; and for processed tomatoes <2 (7 g/day), 2 – <4 (14 g/day), and ≥4 sauce spoons/week.

Multivariate linear regression models were constructed to quantify the association between plasma levels of IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 (as continuous dependent variables in separate models) and dietary factors (as continuous independent variables). Data on IGFBP-1 concentrations were ln(concentration + 1)-transformed, to normalize the distribution of this variable. All linear models have been adjusted for age, BMI, physical activity score, time since last meal or drink, and total energy intake. Since IGF-I and IGFBP-3 are known to be strongly co-regulated, models for IGF-I plasma levels were also adjusted for IGFBP-3 plasma levels, and vice versa. As the regression coefficients and the mean plasma concentrations in dietary categories led to similar conclusions, we have chosen to present only the results of the regression analyses.

The regression coefficients obtained from the regression models were expressed as changes per serving increment (Δ's). The serving increments used were defined as the difference between the tertile or category cut-off points (as described above), for pre- and postmenopausal women separately. Due to very low intakes and limited variation in intakes of total phytoestrogens, total isoflavones, and plant lignans, a serving increment of 1 mg/day was used. P-values below 0.05 were considered to be statistically significant. The SPSS version 10.0 package was used for statistical analyses.

Results

Basic characteristics of our study population are described in **Table 3.1**. IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations were within the normal range. IGF-I concentrations were slightly higher for premenopausal than for postmenopausal women. As described in our previous studies, plasma concentration of IGF-I was strongly positively correlated to the concentration of IGFBP-3 (Pearson correlation coefficient (r) = 0.58 and r = 0.60; $p < 0.001$) (39;40). Similarly, plasma concentrations of IGFBP-1 and IGFBP-2 were positively correlated (r = 0.37 and r = 0.30; p = 0.01) (39;40). Dietary intakes of phytoestrogens and related foods, and lycopene and tomatoes were relatively low.

Table 3.1. Selected characteristics of pre- and postmenopausal women, Prospect-EPIC, 1993-1997

Variable (unit)	Premenopausal (n=224)	Postmenopausal (n=162)
	Median (interquartile range[a])	Median (interquartile range[a])
General characteristics		
Age (years)	50 (49 - 51)	60 (55 - 65)
Height (cm)	165 (161 - 169)	164 (160 - 168)
Weight (kg)	67 (60 - 77)	68 (63 - 77)
Body mass index (kg/m^2)	25 (22 - 27)	26 (23 - 29)
Macronutrients		
Total energy (kJ/day)	7654 (6330 - 8909)	7556 (6527 - 8924)
Total protein (g/day)	70.5 (59.9 - 84.1)	73.0 (62.0 - 86.2)
Animal protein (g/day)	46.0 (36.9 - 55.8)	47.9 (38.9 - 58.6)
Plant protein (g/day)	24.3 (20.4 - 28.5)	24.4 (20.2 - 29.1)
Alcohol (g/day)	8.5 (1.8 - 20.0)	2.8 (0.2 - 12.4)
Food groups		
Soy products (g/day)	0.0 (0.0 - 1.9)	0.0 (0.0 - 1.4)
Legumes (g/day)	7.0 (3.2 - 13.6)	8.3 (3.8 - 13.9)
Cereals (g/day)	162 (121 - 208)	143 (113 - 187)
Tomatoes, raw (g/day)	5.7 (2.8 - 10.5)	3.7 (1.6 - 7.4)
Tomatoes, processed (g/day)	10.3 (4.4 - 18.6)	5.2 (1.0 - 13.2)
Micronutrients		
Total phytoestrogens (mg/day)	0.86 (0.48 - 1.12)	0.77 (0.42 - 1.06)
Total isoflavones (mg/day)	0.15 (0.09 - 0.24)	0.14 (0.09 - 0.23)
Plant lignans (mg/day)	0.63 (0.30 - 0.87)	0.54 (0.25 - 0.86)
Lycopene (mg/day)	3.02 (1.81 - 4.45)	2.17 (1.10 - 3.71)
IGF plasma concentrations		
IGF-I (ng/ml)	150.3 (116.7 - 184.5)	148.3 (112.6 - 175.0)
IGFBP-1 (ng/ml)	14.5 (7.8 - 29.6)	11.6 (7.4 - 22.9)
IGFBP-2 (ng/ml)	400.0 (259.3 - 574.3)	394.8 (281.6 - 562.9)
IGFBP-3 (ng/ml)	3057.7 (2718.8 - 3393.2)	3155.7 (2861.9 - 3599.9)

[a] Interquartile range (p25 – p75).

Pearson correlation coefficients between dietary factors and plasma IGF-I, IGFBP-1, IGFBP-2 and IGFBP-3 ranged from –0.14 to 0.20, and –0.13 to 0.18 for pre- and

postmenopausal women, respectively (data not shown). Statistically significant correlations were observed for raw tomatoes and IGFBP-1 (r = 0.16; p = 0.02), animal protein and IGFBP-2 (r = -0.14; p = 0.04), and soy products and IGFBP-2 (r = 0.20; p = 0.002) in premenopausal women. For postmenopausal women, only plant lignans were statistically significantly correlated with IGFBP-1 (r = 0.18; p = 0.02).
Tables 2, 3, and 4 present the results of the multivariate linear regression analyses. No significant associations between plasma IGF-I and IGFBPs, and total energy and protein (total, animal, plant) intake in both pre- and postmenopausal women were observed (**Table 3.2**).

Table 3.2. Changes (Δ) in plasma IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 (ng/ml) for a specified increment of macronutrient intake in 224 premenopausal and 162 postmenopausal women, separately[a]

Dietary factors	Serving increment[b]	IGF-I[c]		IGFBP-1[d]		IGFBP-2		IGFBP-3[c]	
		Δ (ng/ml)	p^e	Δ (ng/ml)	p^e	Δ (ng/ml)	p^e	Δ (ng/ml)	p^e
Premenopausal women									
Energy	1735 kJ/day	1.6	0.54	-0.8	0.24	-6.1	0.59	12.6	0.57
Total protein[f]	5.9 g/day	0.1	0.96	0.9	0.17	-5.6	0.52	9.3	0.58
Animal protein[f]	8.1 g/day	1.5	0.52	0.8	0.20	-8.0	0.45	27.5	0.17
Plant protein[f]	3.4 g/day	3.2	0.14	-0.1	0.87	4.0	0.67	-35.3	0.05
Alcohol	18.6 g/day	-9.4	0.04	0.2	0.86	-27.3	0.18	90.7	0.02
Postmenopausal women									
Energy	1586 kJ/day	-1.9	0.51	0.04	0.96	6.7	0.59	0.4	0.99
Total protein[f]	8.7 g/day	3.2	0.24	1.1	0.09	-9.0	0.45	-7.6	0.78
Animal protein[f]	8.8 g/day	2.0	0.43	0.7	0.28	-14.3	0.19	1.3	0.96
Plant protein[f]	4.0 g/day	1.9	0.50	0.8	0.26	18.4	0.13	-20.6	0.46
Alcohol	18.6 g/day	-10.0	0.08	-2.8	0.04	-16.2	0.52	111.0	0.06

[a] Adjusted for age, BMI, physical activity score, time since last meal or drink, total energy intake; [b] Increments are calculated as the difference between the extreme cut points of tertiles or relevant categories. The number of pre- and postmenopausal women in the lowest, intermediate, and highest category, respectively were for alcohol (pre: 51, 117, 56; post: 67, 45, 20); [c] IGF-I and IGFBP-3 are mutually adjusted for each other; [d] The concentrations of IGFBP-1 have been ln-transformed (ln(1+conc)) and back-transformed values are reported; [e] Change in plasma levels (ng/ml), p-value; [f] Residual + total energy intake in linear regression model. R^2 of the full models ranged from 34% to 43% for IGF-I, 19% to 20% for IGFBP-1, 17% to 18% for IGFBP-2, and 36% to 40% for IGFBP-3.

Alcohol intake was inversely associated with plasma IGF-I and positively associated with plasma IGFBP-3 in premenopausal women. For example, an increase in alcohol intake of 18.6 g/day (moving from the lowest tertile cut-off point (1 glass/week=1.4 g/day) to the highest tertile cut-off point (2 glasses/day=20 g/day), is associated with a 6% decrease in IGF-I (-9.4 ng/ml; p = 0.04) and a 3% increase in IGFBP-3 (90.7 ng/ml; p = 0.02). In postmenopausal women, increased alcohol intake was associated with a 20% decrease in plasma IGFBP-1 concentrations (-2.8 ng/ml; p = 0.04).
Of the phytoestrogens and related foods (**Table 3.3**), increased soy intake was associated with increased plasma IGFBP-2 concentrations in premenopausal women (11 ng/ml (3%)

increase in IGFBP-2 with each 2.5 g/day increase in soy intake; p = 0.04). In postmenopausal women, intake of plant lignans was positively associated with plasma IGFBP-1 concentrations (example: increase of 5.8 ng/ml (59%) in IGFBP-1 with an increase in plant lignans intake from 0 to 1 mg/day, p = 0.02). No statistically significant associations were observed for phytoestrogens or related foods and IGF-I or IGFBP-3 concentrations.

Table 3.3. Changes (Δ) in plasma IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 (ng/ml) for a specified increment of phytoestrogen related foods and micronutrients intake in 224 premenopausal and 162 postmenopausal women, separately[a]

Dietary factors	Serving increment[b]	IGF-I[c]		IGFBP-1[d]		IGFBP-2		IGFBP-3[c]	
		Δ (ng/ml)	p[e]	Δ (ng/ml)	p[e]	Δ (ng/ml)	p[e]	Δ (ng/ml)	p[e]
Premenopausal women									
Soy products	2.5 g/day	-0.1	0.95	0.01	0.98	10.6	0.04	-12.5	0.19
Legumes	7 g/day	-1.5	0.52	0.1	0.93	7.0	0.50	-9.2	0.64
Cereals	57 g/day	3.6	0.26	-1.7	0.05	-16.2	0.26	-9.1	0.74
Total phytoestrogens	1 mg/day	-0.5	0.75	-0.3	0.42	9.4	0.17	-0.4	0.98
Total isoflavones	1 mg/day	-0.3	0.85	-0.3	0.46	9.2	0.20	-3.4	0.80
Plant lignans	1 mg/day	-8.4	0.38	-1.5	0.56	14.6	0.42	105.6	0.19
Postmenopausal women									
Soy products	2.5 g/day	-0.1	0.96	-0.2	0.53	10.8	0.07	-8.1	0.56
Legumes	7 g/day	1.9	0.37	0.1	0.92	0.1	0.99	-20.1	0.34
Cereals	45 g/day	3.1	0.33	1.2	0.14	7.3	0.60	-3.3	0.92
Total phytoestrogens	1 mg/day	0.6	0.80	-0.1	0.80	6.6	0.53	3.1	0.90
Total isoflavones	1 mg/day	0.5	0.85	-0.5	0.41	5.8	0.59	0.8	0.98
Plant lignans	1 mg/day	2.9	0.79	5.8	0.02	24.7	0.59	45.9	0.66

[a] Adjusted for age, BMI, physical activity score, time since last meal or drink, total energy intake; [b] Increments are calculated as the difference between the extreme cut points of tertiles or relevant categories. The number of pre- and postmenopausal women in the lowest, intermediate, and highest category, respectively were for soy (pre: 127, 55, 42; post: 105, 31, 26), and for legumes (pre: 111, 60, 53; post: 72, 50, 40); [c] IGF-I and IGFBP-3 are mutually adjusted for each other; [d] The concentrations of IGFBP-1 have been ln-transformed (ln(1+conc)) and back-transformed values are reported; [e] Change in plasma levels (ng/ml), p-value; R^2 of the full models ranged from 34% to 42% for IGF-I, 19% to 20% for IGFBP-1, 17% to 19% for IGFBP-2, and 36% to 39% for IGFBP-3.

For intake of lycopene and tomatoes, no statistically significant associations were found with plasma components of the IGF-system (**Table 3.4**).

Discussion

In our population of Dutch pre- and postmenopausal women, no statistically significant associations were found between energy and protein intake and IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 plasma concentrations. Similarly, within the relatively low intakes of phytoestrogens and lycopene in our population, no significant associations of these compounds with plasma concentrations of IGF-I and IGFBP-3 were observed. However,

Table 3.4. Changes (Δ) in plasma IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 (ng/ml) for a specified increment of raw tomatoes, cooked tomatoes, and lycopene intake in 224 premenopausal and 162 postmenopausal women, separately[a]

Dietary factors	Serving increment[b]	IGF-I[c]		IGFBP-1[d]		IGFBP-2		IGFBP-3[c]	
		Δ (ng/ml)	p^e	Δ (ng/ml)	p^e	Δ (ng/ml)	p^e	Δ (ng/ml)	p^e
Premenopausal women									
Raw tomatoes	5 g/day	-3.8	0.09	1.1	0.09	-3.6	0.72	21.6	0.25
Processed tomatoes	7 g/day	1.4	0.55	-0.3	0.63	-12.5	0.21	-7.6	0.69
Lycopene	1.86 mg/day	0.3	0.90	0.5	0.53	-11.9	0.33	-16.6	0.47
Postmenopausal women									
Raw tomatoes	5 g/day	2.7	0.34	-0.1	0.91	-2.8	0.82	-30.3	0.28
Processed tomatoes	7 g/day	-0.7	0.80	-0.4	0.54	-20.4	0.11	23.8	0.41
Lycopene	1.65 mg/day	0.3	0.89	-0.4	0.46	-10.4	0.31	7.0	0.77

[a] Adjusted for age, BMI, physical activity score, time since last meal or drink, total energy intake; [b] IGF-I and IGFBP-3 are mutually adjusted for each other; [c] The concentrations of IGFBP-1 have been ln-transformed (ln(1+conc)) and back-transformed values are reported; [d] Increments are calculated as the difference between the extreme cut points of tertiles or relevant categories. The number of pre- and postmenopausal women in the lowest, intermediate, and highest category, respectively were for raw tomatoes (pre: 99, 64, 61; post: 102, 28, 32); and for processed tomatoes (pre: 80, 61, 83; post: 93, 34, 35); [e] Change in plasma levels (ng/ml), *p*-value; R^2 of the full models ranged from 34% to 42% for IGF-I, 19% to 20% for IGFBP-1, 17% to 18% for IGFBP-2, and 36% to 39% for IGFBP-3.

intakes of some (sources of) phytoestrogens were significantly positively associated with IGFBP-1 or –2. Furthermore, higher alcohol intake was significantly associated with lower IGF-I and higher IGFBP-3 plasma concentrations in premenopausal women, and with lower plasma IGFBP-1 concentrations in postmenopausal women. As we studied several dietary factors in relation to four IGF-system components, we cannot exclude that some of our findings may be due to chance.

Elevated serum IGF-I concentrations have been associated with increased cancer risk (2-8), however, the association of IGFBP-3 concentrations with cancer risk remains controversial. Some studies have shown an increase in risk for higher IGFBP-3 concentrations (3;5;8), others have observed a decrease in risk (2;6;7). In epidemiological studies, the IGF-I/IGFBP-3 molar ratio has been used as an approximate index of 'free', bioactive IGF-I, since IGFBP-3 is the main binding protein of IGF-I in the circulation. However, the biological effects of the different IGF binding proteins on IGF-I bioactivity are still relatively unknown. Similar to IGFBP-3, IGFBP-1 and –2 may also reduce bioactive IGF-I by binding to it and making it unavailable for the IGF-I receptor. On the other hand, IGFBP-1 and –2 allow the transport of IGF-I out of the bloodstream, which may result in increased IGF-I concentrations at the tissue level. Since it is unknown which is the relevant measure of bioactive IGF-I, we did not use a proxy such as the IGF-I/IGFBP-3 molar ratio.

Energy balance, dietary energy intake, and to a lesser extent protein intake, may be linked to cancer risk (47;48) and have been implicated in the modulation of the IGF-system. Experimental studies in animals and humans have shown that severe dietary

energy and protein restriction (e.g. fasting) results in an acute decrease in IGF-I, acute increase in IGFBP-1, chronic increase in IGFBP-2, and chronic decrease in IGFBP-3 (11). Both energy and protein are needed to restore IGF-I after fasting (11). Our results, in a study population with adequate intake of energy and protein, are consistent with most previous cross-sectional studies, in which no association between energy and protein intake and circulating IGF-I and IGFBP-3 was observed (12-20). The two largest cross-sectional studies did show a positive association between IGF-I and protein intake within the normal range (22;23), as is also found by two smaller studies (21;24). However, with respect to protein intake and IGFBP-3 concentrations, and energy intake and IGF-I and IGFBP-3 concentrations the two largest studies are also inconsistent (22;23). Taken together, these results suggest that although major changes in energy and protein intake do modulate the IGF-system, the relatively minor differences observed in the general population probably do not play an important role.

Alcohol consumption has been associated with increased risk of various types of cancer (49). For instance, risk of breast cancer is increased by moderate consumption of alcohol (50;51), but not further increased by heavy drinking (i.e., at least 60 g/day of alcohol) (51). A possible biological mechanism for this association could be that moderate alcohol consumption increases IGF-I production by the liver, which is not further increased in heavy drinkers due to alcohol-induced liver damage (25). In our study, however, higher alcohol intake was associated with decreased IGF-I and increased IGFBP-3 plasma concentrations in premenopausal women. We observed these associations only when IGF-I and IGFBP-3 were mutually adjusted for each other, suggesting departure from the usual concurrent hepatic IGF-I and IGFBP-3 stimulus. We also found that higher alcohol intake was associated with decreased IGFBP-1 concentrations in postmenopausal women. Cross-sectional studies on the association between alcohol intake and the IGF-system published to date have not investigated IGFBP-1, and have been very inconsistent with respect to IGF-I and IGFBP-3 (14;15;17;22;26). This may be partly explained by the different ranges of intakes studied. The relevance of our findings remains unclear and needs further investigation.

Our assessment of alcohol, total energy, and protein intake has previously been shown to be reproducible (43). However, the food frequency questionnaire (FFQ) we used has been shown to underestimate total energy and protein intake as compared to basal metabolic rate and urinary nitrogen excretion used as reference methods, respectively. The underestimation of protein intake by the FFQ was more pronounced for relatively higher intakes. This might have led to an underestimation of a possible association between protein intake and circulating IGF system components. However, this is not thought to have markedly influenced our results, since protein intake in our study population is relatively low.

Besides energy and macronutrient intake, micronutrients or other bioactive compounds in the diet may also influence the IGF-system. Phytoestrogens are plant substances that structurally resemble endogenous estradiol and are thought to interact with the estrogen

receptor. Selective estrogen receptor modulators (e.g. tamoxifen, raloxifene) have been shown to decrease circulating IGF-I levels in previous human studies (27-29), and may also increase some IGFBP levels (27;28). Additionally, studies in mouse models showed a reduction of serum IGF-I or IGF-I mRNA expression in breast or prostate tissue by phytoestrogens (52-54).

In our cross-sectional study, with limited variation in intake of phytoestrogens and related foods, no association with IGF-I and IGFBP-3 was observed. Two recent cross-sectional studies in an Asian population with high variation in intake also did not observe any association in women (19;20), but found a positive association between soy intake and circulating IGF-I in men (20). Interestingly, we did find that a higher soy consumption was associated with increased plasma IGFBP-2 concentrations, and a higher intake of plant lignans with increased IGFBP-1 concentrations. This could be in line with the increased IGFBP-1 and –2 concentrations observed in vegan women, who usually have a relatively high consumption of phytoestrogens, as compared to vegetarians and meat eaters (17). Four human intervention studies investigated the effect of soy protein intake with high isoflavones compared to soy or milk protein with low or no isoflavones, on serum IGF-I (31-33;55). The results from these studies were inconsistent, and may point to opposing effects of soy protein and phytoestrogens, with IGF-I being increased by soy protein, but decreased by isoflavones. Additionally, this association may well be modified by endogenous estrogen levels, resulting in different associations for men and women, and for pre- and postmenopausal women.

Another component of the diet which may be related to the IGF-system is lycopene, an anti-oxidant mainly found in tomatoes. Tomato and lycopene intake have been associated with decreased risk of several types of cancer and possibly the IGF-system is the intermediate in this association (56). In our study, no association between either tomatoes or lycopene and IGF-I or IGFBP-3 was observed. Four cross-sectional studies have investigated the relationship between tomato consumption (14;15;18) or lycopene intake (22) and IGF-I and IGFBP-3. In three of these studies, higher intake of cooked or processed tomatoes or lycopene was associated with either lower IGF-I levels (14), higher IGFBP-3 levels (22), or a lower IGF-I/IGFBP-3 molar ratio (18). Two in vitro studies have shown that lycopene can inhibit IGF-I-stimulated growth of endometrium and mammary cancer cell lines (34;35). In these studies, lycopene reduced IGF-I receptor signaling, and increased levels of membrane-associated IGFBPs (35). Lycopene supplementation also increased plasma IGFBP-3 concentrations in ferrets (36). In a small human intervention study, IGF-I was decreased both in the lycopene intervention as in the control group (57). Overall, these studies suggest that lycopene might influence the IGF-system both by decreasing IGF-I and increasing IGFBPs.

We hypothesized that high intakes of phytoestrogens, lycopene, and related foods were associated with decreased IGF-I and increased IGFBP-3 concentrations, which we could not confirm in the current cross-sectional study. Several methodological aspects of our study may have contributed to this. The FFQ used for the assessment of phytoestrogens and

related foods, and lycopene and tomato intake, was not specifically designed for this purpose. Although the FFQ contained most of the important food sources of phytoestrogens (tempeh, tofu, vegetarian burgers, and legumes) and lycopene (pasta sauce, raw tomatoes, baked/fried tomatoes), other food items that may have contributed to phytoestrogen and lycopene intake were not included in the questionnaire (e.g. soy milk, tomato soup). Therefore, the true intake of phytoestrogens and lycopene in our population may be underestimated. Overall, intake of phytoestrogens and lycopene in our study is relatively low, but comparable to that observed in many other Western populations (44;58-60). Within this low range, limited variation in phytoestrogens and related foods, and lycopene and tomato intake might explain why we did not observe an association with IGF-I and IGFBP-3. Additionally, much higher phytoestrogen and lycopene intakes may be needed to affect the IGF-system. However, it is also possible that phytoestrogens and lycopene mainly influence IGFBP-1 and IGFBP-2 levels, and do not have a direct effect on IGF-I and IGFBP-3, as is also suggested by our results.

Some general remarks can be made about the design of our study. The association between diet and the IGF-system might differ by gender, menopausal status, and menstrual cycle phase, as the IGF-system is known to be strongly interrelated with estrogen action. Therefore, we studied pre- and postmenopausal women separately, although we were not able to adjust for menstrual cycle phase. As the premenopausal women in our study were relatively old, they may not be comparable to premenopausal women in general. However, our study is a good reflection of a Western population of women over 50 with respect to IGF levels (4;7;8) and dietary intake. With respect to the relevant time frame, it is yet unknown which has the greatest effect on the IGF-system: a high dietary exposure for a short duration or a relatively low dietary exposure for a longer duration, which is the type of exposure we assessed in the current study. Consequently, we do not know whether food intake in the days prior to plasma sampling may have disturbed any association between plasma IGF levels and habitual food intake as assessed by our questionnaire.

In conclusion, in the current study the two IGF binding proteins, IGFBP-1 and IGFBP-2, which have received relatively little attention in epidemiological studies so far, were associated with the consumption of alcohol and some measures of phytoestrogen intake. The relevance of changes in plasma concentrations of these binding proteins, also with respect to IGF-I bioactivity, remains controversial and deserves further investigation. Besides alcohol, we found no dietary determinants of IGF-I and IGFBP-3 in our study. It would be interesting to investigate these dietary factors in relation to the IGF-system in populations with a higher consumption and greater variation in consumption of phytoestrogens or lycopene (e.g. Asian or Italian populations). Human dietary intervention studies would ultimately provide the answers with respect to the effects of these dietary compounds on the different circulating IGF components.

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Part 1
DIETARY INTERVENTION STUDIES

Effects of lycopene on the Insulin-like Growth Factor (IGF) system in premenopausal breast cancer survivors and women at high familial breast cancer risk

4

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Nutrition and Cancer, in press

Abstract

IGF-I is an important growth factor associated with increased risk of premenopausal breast cancer. We conducted a randomized, placebo-controlled, double-blind, cross-over trial to evaluate whether tomato-derived lycopene supplementation (30 mg/d for two months) decreases serum levels of total IGF-I in in premenopausal women with 1) a history of breast cancer (n = 24), or 2) a high familial breast cancer risk (n = 36). Also, IGFBP increasing effects were evaluated. Lycopene supplementation did not significantly alter serum total IGF-I and other IGF system components in the two study populations combined. However, statistically significant discordant results were observed between the two study populations (i.e., $p < 0.05$ for total IGF-I, free IGF-I and IGFBP-3). Total IGF-I and IGFBP-3 were increased in the breast cancer survivor population (total IGF-I, 7.0%, 95%CI: -0.2 to 14.3%; IGFBP-3, 3.3%; 95%CI: 0.7 to 6.0%), and free IGF-I was decreased in the family history population (-7.6%; 95%CI: -14.6 to -0.6%). This randomized controlled trial shows that two months of lycopene supplementation has no effect on serum total IGF-I in the overall study population. However, lycopene effects were discordant between the two study populations showing beneficial effects in high-risk healthy women but not in breast cancer survivors.

Introduction

Extensive review and meta-analyses of epidemiological studies have recently confirmed that the risk of several cancers (e.g. prostate, breast, colorectal) is increased in individuals with relatively high serum concentrations of Insulin-like Growth Factor (IGF)-I (1;2). In several studies the increased risk of breast cancer was limited to premenopausal women (3-11). Interestingly, women with a family history of breast cancer were found to have higher serum IGF-I levels as compared to women without a family history (12).

Experimental studies show that at the tissue level, IGF-I and IGF-II are potent mitogenic and anti-apoptotic factors, stimulating cell proliferation and tumor growth. The strongest evidence for a causal association between circulating IGF-I concentrations and cancer risk comes from animal models. In LID mice the *igf1* gene is deleted exclusively in the liver, leading to dramatically reduced serum levels of IGF-I (75% decrease) but normal growth and development (13). These mice display delayed onset of chemically and genetically induced mammary tumors (14). In another mouse model (p53-deficient mice) energy restriction induced a less dramatic reduction in serum IGF-I (25% decrease), which also decreased malignant transformation (15). Furthermore, in experimental models the growth of many established cancers can be inhibited by pharmacological strategies that reduce IGF-I receptor signaling (16).

Both epidemiological and experimental evidence point towards the IGF system as a potential target for cancer prevention. Cross-sectional studies in humans suggested an association between higher lycopene intake and/or tomato consumption and lower circulating levels of (bioavailable) IGF-I (2). Several studies reported that women with relatively high levels of lycopene in serum or adipose tissue show a decreased risk of breast cancer (17-19), although two recent studies could not confirm these findings (20;21). Lycopene was found to interfere with the proliferative activity of the IGF system in cell cultures by increasing amounts of membrane-associated IGF binding proteins (22;23). In animal models, lycopene upregulated circulating IGFBP-3 levels and downregulated IGF-I expression in the prostate (24-26). So far, only three small intervention studies have investigated the effect of lycopene on the IGF system in humans, suggesting only small IGF-I lowering effects if any (27-29).

We conducted a randomized controlled cross-over trial to evaluate the effect of 2 months of tomato-derived lycopene supplementation on serum IGF system components in premenopausal women at increased risk of breast cancer.

Material and Methods

Study population and recruitment

The study protocol was approved by the Ethical Committee of the Netherlands Cancer Institute – Antoni van Leeuwenhoek hospital. We recruited participants who were or had been regularly screened at this hospital. Two study populations at increased risk of breast cancer (premenopausal women, ≤ 50 years of age) were included: 1) women with a history

of breast cancer diagnosed ≥ 4 years before study entry ('breast cancer survivor population'), and 2) healthy women with a family history of breast cancer, i.e., at least 1 first degree family member with breast cancer diagnosed before age 50, or carriers of a mutation in the breast cancer susceptibility genes BRCA1 or BRCA2 ('family history population'). Study participants were selected on basis of the criteria listed in **Table 4.1**. Potentially eligible individuals (*n* = 339) were invited by mail to participate in the study, after which further exclusion criteria were checked by phone (see Table 4.1). All participants were recruited between February 2003 and January 2005. Written informed consent was obtained from all participants before entry into the trial.

Table 4.1. Eligibility criteria for study participants

Breast cancer survivor population	Family history population
Selection criteria - based on medical records	
Age ≤50 yrs at start of study	
Diagnosed with breast cancer >4 yrs ago	No malignancies
No other malignancies before/after BC	
No distant metastases at time of diagnosis	Known BRCA1/2 mutation carrier or
No chemo- or endocrine therapy[a]	Family history (≥1 first degree family member
No local/regional/distant recurrences	diagnosed with BC at age ≤ 50)
No prophylactic mastectomy of the contralateral breast	No prophylactic mastectomy
No (metabolic) comorbidities (diabetes, acromegaly, etc)	
Exclusion criteria - checked with participant by phone	
Peri- or postmenopausal (<10 menses in past 12 months)	
Irregular menstrual cycles	
Comorbidity (diabetes, specific food allergies, etc)	
Pregnant or intention to become pregnant within 6 months	
Use of dietary supplements containing lycopene of isoflavones	

[a] one participant received chemotherapy 19 years previously, which had not influenced menstrual cycles

Study design

We conducted a randomized, placebo-controlled, double-blind, cross-over study. Participants were randomized into two groups stratified by subpopulation ('breast cancer survivor population' and 'family history population'). Subjects were allocated to receive lycopene capsules in the first and placebo capsules in the second intervention period (LP) or vice versa (PL), according to a randomization scheme with permuted blocks. The duration of each intervention period and wash-out period was approximately two months (i.e., two menstrual cycles, exact length in days depended on individual menstrual cycles). Randomization and supplement allocation were performed by the hospital pharmacy, such that both participants and study personnel were blinded to the group allocation.

The lycopene capsules (Lyc-o-Mato® 6%, LycoRed Ltd., Beer-Sheva, Israel) contained ~15

mg/capsule of total lycopene. Lyc-o-Mato is a tomato extract (oleoresin) containing 6% lycopene as well as other natural tomato phytonutrients (i.e., 1.5 mg phytoene, 1.4 mg phytofluene, 0.4 mg beta-carotene, 5 mg tocopherols) extracted from lycopene rich tomatoes. Placebo and lycopene capsules were identical in appearance. Participants were asked to take two capsules per day (total dose: 30 mg lycopene/day), one with breakfast and one with dinner. This dose of lycopene is known to result in maximal serum levels of lycopene, where relevant benefits can be expected, but is within the range that can be consumed through the diet. Compliance was measured by returned capsule counts, recording of taken capsules in a daily notebook, and measurement of serum lycopene levels at the beginning and end of both intervention periods. Participants were asked to maintain their habitual diet and lifestyle.

The sample size calculation for this randomized controlled trial was based on a pilot study in 6 individuals in which we observed a within-person coefficient of variation of 11% in IGF-I concentration in multiple serum samples drawn 3-12 months apart. This resulted in a required sample size of 26 participants to detect a 10% difference in serum IGF-I between the treatment groups with 90% power (2-sided α = 0.05).

Study procedures

Subjects visited the hospital at the beginning and end of both intervention periods. All four visits were planned to take place during days 3 to 5 of the menstrual cycle, when estradiol and progesterone levels are relatively stable. During each study visit an overnight fasting blood sample (30 ml) was drawn and body weight and waist and hip circumference were measured. Body height was measured during the first visit.

To assess habitual diet of the study population, a 24-h recall was conducted at each study visit. The method of interviewing and coding of foods and portion sizes was standardized, and was performed by trained nutritionists and graduate students in nutrition. Energy and nutrient intakes were calculated using the VBS food calculating system (BAS Nutrition Software) based on the Dutch food composition table (30). Habitual physical activity over the two months preceding each visit was assessed using the validated self-administered Short Questionnaire to Assess Health-enhancing physical activity (SQUASH) (31). During both intervention periods, participants kept a daily notebook in which they recorded information about illness and medical/physical complaints, medicine and hormone use, menstrual cycle, smoking, and consumption of products rich in lycopene (tomato products, specific fruits). Menstrual cycle length, use of hormonal preparations, and smoking habits at baseline were assessed using a general questionnaire.

Laboratory methods

Serum and EDTA-plasma samples, obtained at all four time points, were frozen and stored at -30/-80°C until further analysis. Serum total IGF-I, plasma free IGF-I, and serum IGFBP-1, IGFBP-2, and IGFBP-3 levels were measured in samples drawn at the end of each intervention period, and were determined as previously described using IRMA (free and

total IGF-I) and RIA (IGFBP-1, -2, -3) (32).
The serum concentration of lycopene was determined at the beginning and end of both intervention periods using HPLC with detection at 472 nm, according to the method described by Gueguen et al. (33). Samples were kept in the dark and at sub-ambient temperature until analysis. HPLC separation was achieved on a glass column (10 cm x 3 mm; ID) packed with Nucleosil C18 material using a mobile phase consisting of methanol:acetonitrile:tetrahydrofuran (75:20:5; v/v/v) delivered at a flow rate of 0.4 ml/min.
Serum estradiol (E2) and sex hormone-binding globulin (SHBG) were measured using immunoassays based on the electrochemiluminescence principle. Both assays were used on the E170 (Elecsys module) immunoanalyzer (Roche Diagnostics, Mannheim, Germany).

Statistical analyses
All statistical analyses were conducted based on the 'intention to treat' principle, including all participants who were randomized and donated a blood sample on all four study visits, irrespective of compliance to the intervention protocol. As a secondary approach 'per protocol' analyses were conducted, excluding all participants who were non-compliant, i.e., less than 80% of capsules taken (based on returned capsules count) and/or less than 20% increase in serum lycopene concentration after lycopene intervention.
The main parameter of interest, on which the sample size and power calculations were based, was the relative cross-over difference in total IGF-I (i.e., concentration after lycopene intervention minus concentration after placebo treatment, expressed as percentage change relative to the concentration after placebo treatment). Analyses were conducted for the two study populations combined as well as for each separately. Relative cross-over differences in serum concentrations of free IGF-I, IGFBP-1, IGFBP-2, and IGFBP-3 were secondary endpoints. A one-sample t-test was conducted to evaluate whether the mean relative cross-over differences deviated from null. For parameters that were not normally distributed (i.e., IGFBP-1) a non-parametric sign test was performed. Absolute differences between serum concentrations after lycopene and after placebo were tested using paired t-tests (total IGF-I, IGFBP-2, IGFBP-3, SHBG, lycopene) or Wilcoxon signed ranks tests (if not normally distributed, i.e., free IGF-I, IGFBP-1, estradiol). To test whether lycopene effects differed between the two study populations independent samples t-tests or Wilcoxon rank sum tests were performed. P-values were determined by two-sided tests, and differences were considered to be statistically significant at p-values lower than 0.05.
Descriptive characteristics were computed for both study populations and for both randomized groups, separately. We calculated whether relevant changes occurred in dietary and lifestyle factors known to influence the IGF system, i.e., dietary intake of macronutrients or products relatively rich in lycopene, body weight, waist and hip circumference, total physical activity score, during the study period for both intervention

groups, separately.

Results

Of 243 eligible women, 75 signed informed consent and were randomized into one of two intervention sequences (**Figure 4.1**). The drop-out rate was 20% (reasons for drop-out, Figure 1), resulting in 60 participants who completed the study protocol. In the 'breast cancer survivor population' the median time since diagnosis of breast cancer was 86 months (range 52 to 200 months).

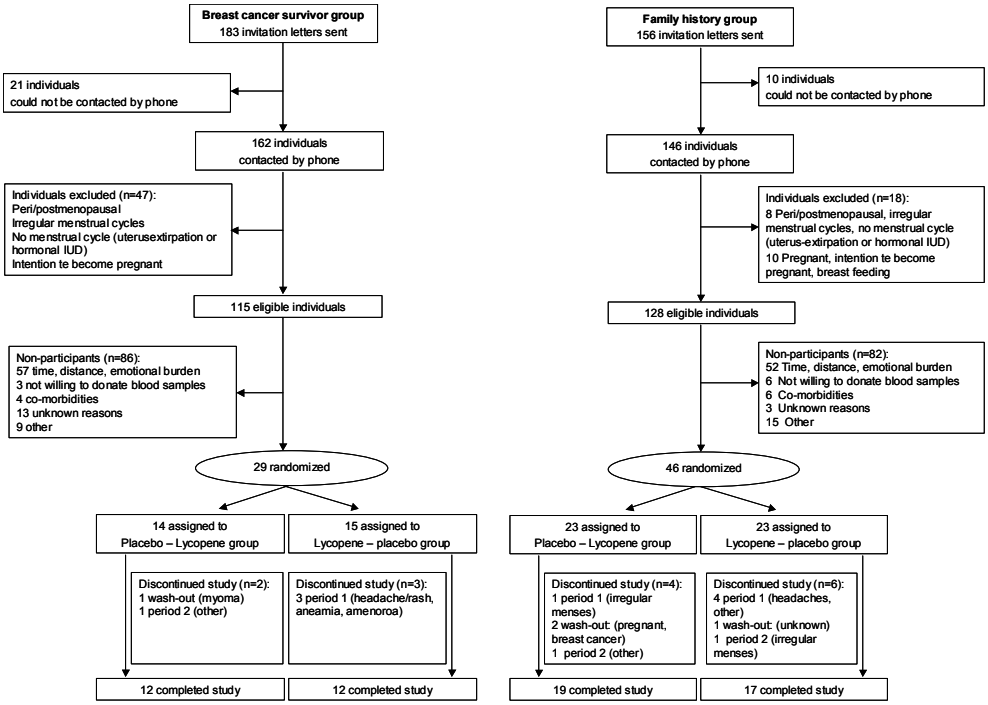


Figure 4.1. Flow chart of participant recruitment.

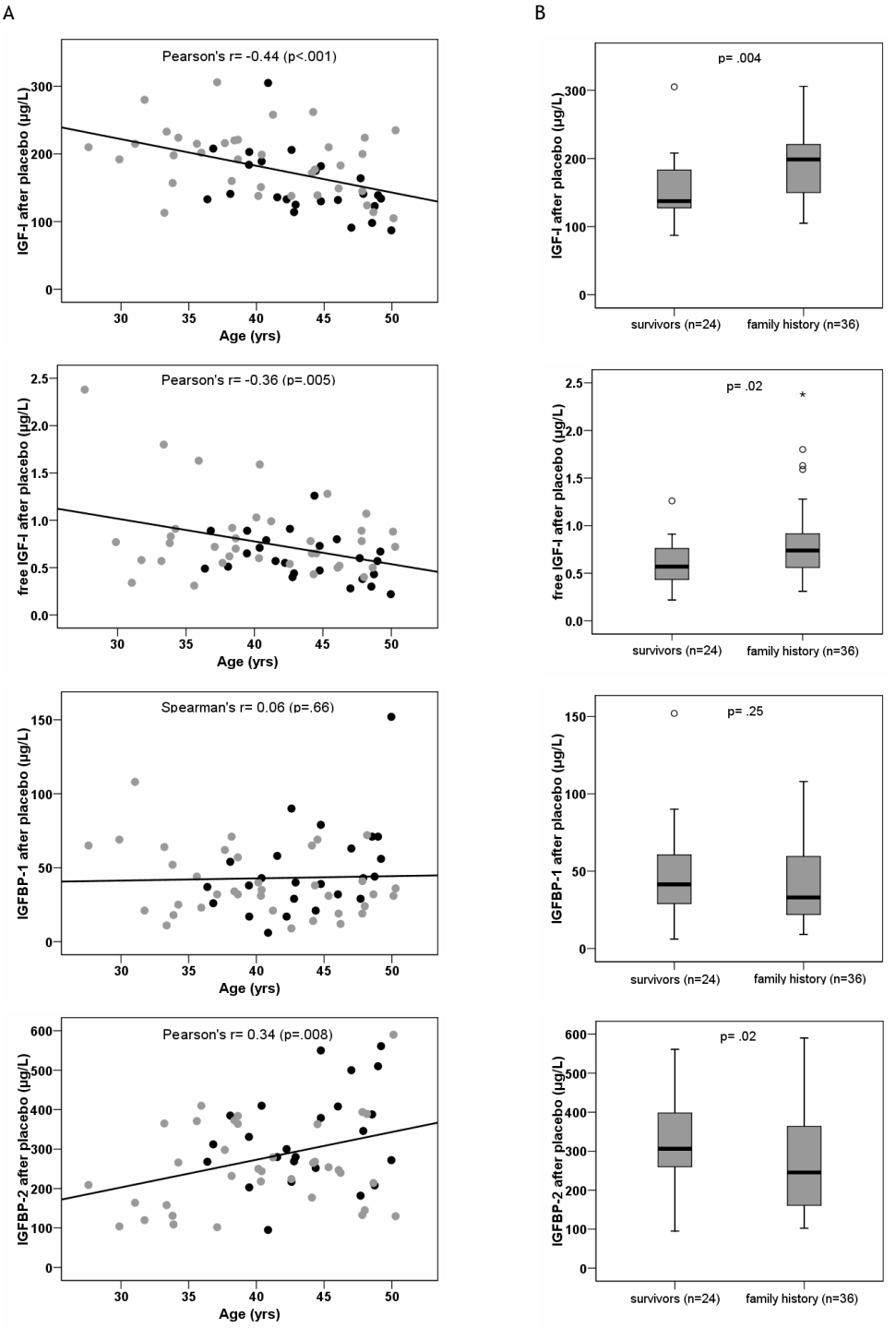
The general characteristics of the study population are shown in **Table 4.2**, for the 'breast cancer survivor population' (n = 24) and the 'family history population' (n = 36), separately. Participants were aged 42 $\pm$ 6 years (mean $\pm$ SD; range 26 to 50 years) at study entry. Baseline body weight, BMI, waist circumference, mean energy and macronutrient intake, and overall physical activity score were comparable in both study groups as well as in the randomized groups. Current oral contraceptive use was observed in only one individual in the 'breast cancer survivor population', whereas almost half of the participants in the 'family history population' were current oral contraceptive users. The

Table 4.2. General characteristics (mean ± sd) of the study populations at baseline

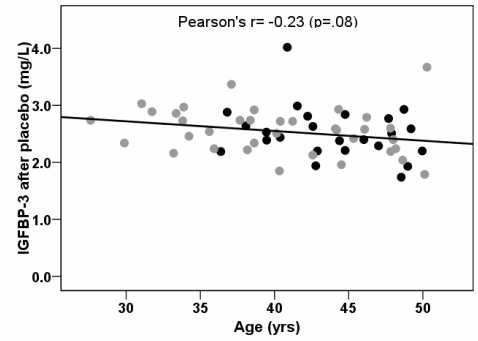
		Breast cancer survivor population		Family history population	
		LP[a] (*n* = 12)	PL[a] (*n* = 12)	LP[a] (*n* = 17)	PL[a] (*n* = 19)
Age, y		44 ± 4	43 ± 5	42 ± 6	39 ± 6
Body height, cm		172 ± 6	170 ± 7	170 ± 6	170 ± 7
Body weight, kg		68 ± 7	71 ± 11	72 ± 11	75 ± 9
BMI, kg/m^2		23 ± 3	25 ± 2	25 ± 4	26 ± 3
Waist circumference, cm		79 ± 5	84 ± 9	81 ± 9	85 ± 8
Energy, kJ/d		8413 ± 1936	8597 ± 1622	8661 ± 2401	8167 ± 3456
Protein, energy%		16 ± 4	16 ± 2	15 ± 4	16 ± 4
Fat, energy%		34 ± 11	35 ± 9	36 ± 11	32 ± 10
Carbohydrates, energy%		44 ± 10	42 ± 9	45 ± 9	48 ± 11
Physical activity, total score		5838 ± 2020	5635 ± 1679	6382 ± 1930	6180 ± 2260
Smoking[b], *n* (%)	Current	3 (25)	2 (17)	2 (12)	6 (35)
	Past	2 (17)	6 (50)	5 (29)	3 (18)
	Never	7 (58)	4 (33)	10 (59)	8 (47)
Oral contraceptives[b], *n* (%)	Current	0	1 (8)	8 (47)	7 (41)
	Past	11 (92)	11 (92)	8 (47)	9 (53)
	Never	1 (8)	0	1 (6)	1 (6)
Hormonal preparations for menopausal symptoms[b], *n* (%)					
	Current	0	0	0	0
	Past	1 (8)	0	0	0
	Never	11 (92)	12 (100)	17 (100)	17 (100)
Parity[b], *n* (%)	0	2 (17)	3 (25)	5 (29)	5 (29)
	1-2	8 (67)	7 (58)	9 (53)	9 (53)
	>2	2 (17)	2 (17)	3 (18)	3 (18)
Menstrual cycle length[b], *n* (%)					
	≤24d	1 (8)	0	1 (6)	1 (6)
	25-26d	2 (17)	2 (17)	2 (12)	3 (18)
	27-29d	5 (42)	1 (8)	3 (18)	2 (12)
	30-31d	3 (25)	8 (67)	11 (65)	10 (59)
	≥32d	1 (8)	1 (8)	0	1 (6)
Family history[b], *n* (%)	Yes	5 (42)	5 (42)	16 (94)	17 (100)
	No	7 (58)	7 (58)	1 (6)	0
Duration lycopene intervention, days		56 ± 6	58 ± 10	55 ± 4	57 ± 10

[a] Group LP: Lycopene – Placebo time sequence, group PL: Placebo – Lycopene time sequence; [b] missing data (no general questionnaire available) in 2 individuals from FAMHIS - group PL.

mean duration of lycopene supplementation was equal among the different groups. Age was significantly inversely correlated with total and free IGF-I ($p < 0.01$) and IGFBP-3 ($p = 0.08$), and positively with IGFBP-2 ($p < 0.01$) measured after placebo treatment (**Figure 4.2A**). In the 'family history population' statistically significant higher concentrations of free and total IGF-I, and lower concentrations of IGFBP-2, were observed as compared to the 'breast cancer survivor population', measured after placebo treatment (**Figure 4.2B**).


A
B
Pearson's r= -0.44 (p<.001)
IGF-I after placebo (µg/L)
Age (yrs)
p= .004
survivors (n=24)
family history (n=36)
Pearson's r= -0.36 (p=.005)
free IGF-I after placebo (µg/L)
p= .02
Spearman's r= 0.06 (p=.66)
IGFBP-1 after placebo (µg/L)
p= .25
Pearson's r= 0.34 (p=.008)
IGFBP-2 after placebo (µg/L)

A *(continued)*



B *(continued)*

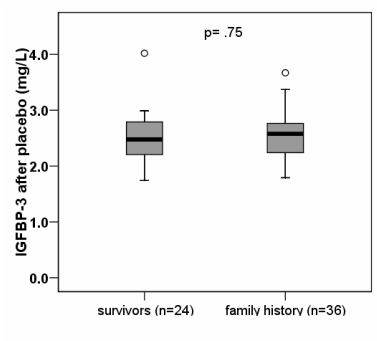


Figure 4.2. Effect of age and study population on the serum concentrations after placebo, of total and free IGF-I and the IGF binding proteins.
Correlation (A) between age and serum concentrations of IGF system components in the breast cancer survivor population (black markers) and the family history population (grey markers).
Serum concentrations (B) of IGF system components after placebo in the breast cancer survivor population and the family history population. Boxplots indicating: median (horizontal bar), interquartile range (box, 25th to 75th percentile), and the range including outliers (° values between 1.5 and 3 box lengths from the 25th and 75th percentile, * values more than 3 box lengths from the 25th and 75th percentile). *P*-values for independent samples t-test (total IGF-I, IGFBP-2 and IGFBP-3) or Wilcoxon rank sum tests (free IGF-I and IGFBP-1).

Total IGF-I concentration after placebo treatment was statistically significantly inversely correlated with concentrations of IGFBP-1 (Spearman correlation coefficient: r = -0.34; p = 0.007) and IGFBP-2 (Pearson r = -0.46; p < 0.001), and positively correlated with IGFBP-3 (Pearson r = 0.63; p < 0.001) and free IGF-1 (Spearman r = 0.43; p = 0.001). In contrast, free IGF-I after placebo treatment was significantly inversely correlated with IGFBP-1 (Spearman r = -0.27; p = 0.04) only, and not with IGFBP-2 and IGFBP-3 (data not shown).
The concentrations of circulating IGF system components after placebo and after lycopene intervention for the total population are shown in **Table 4.3**. No statistically significant differences were observed for total and free IGF-I, nor for any of the IGF binding proteins. The serum lycopene concentration was significantly increased after lycopene supplementation when compared to treatment with placebo (p < 0.001). The changes in serum lycopene as induced during the lycopene intervention period did not significantly correlate with alterations in serum IGF system components (data not shown), except for plasma free IGF-I (Pearson's r = -0.29, p = 0.02). We attempted to minimize any possible effects of changes in the levels of sex steroid hormones on the various components of the IGF system by planning all study visits on days 3 to 5 of the menstrual cycle for each participant. As a consequence, the mean serum levels of estradiol and sex hormone binding globulin (SHBG) did not differ between the lycopene intervention and the placebo period (Table 4.3). As our study population consisted of groups with different personal and

Table 4.3. Serum concentration of IGF system components after lycopene intervention and after placebo in the total study population (n=60)

	Concentration after placebo			Concentration after lycopene			Within-person difference	
	mean ± sd	*median*	*range*	*mean ± sd*	*median*	*range*	*absolute*	*relative*
Total IGF-I (µg/L)	176 ± 51	176	87 - 306	175 ± 48	171	93 - 291	-1	1%
Free IGF-I (µg/L)	0.74 ± 0.38	0.66	0.22 - 2.38	0.70 ± 0.35	0.63	0.26 - 1.89	-0.03	-2%
IGFBP-1 (µg/L)	43 ± 26	38	6 - 152	43 ± 31	39	7 - 177	-2	-4%
IGFBP-2 (µg/L)	285 ± 119	268	95 - 590	282 ± 126	254	96 - 610	-3	-1%
IGFBP-3 (mg/L)	2.54 ± 0.42	2.54	1.74 - 4.02	2.54 ± 0.38	2.51	1.64 - 3.80	0.00	1%
Lycopene (µmol/L)	0.25 ± 0.10	0.25	0.09 - 0.64	0.52 ± 0.19	0.54	0.04 - 0.97	0.26[a]	125%[a]
Estradiol (pmol/L)	195 ± 153	147	50 - 841	205 ± 218	147	55 - 1554	7	8%
SHBG (nmol/L)	76 ± 36	68	24 - 190	78 ± 37	68	23 - 172	2	4%

[a] Absolute or relative within-person cross-over difference between the concentration after placebo and the concentration after lycopene significantly different from zero ($p < 0.05$). Absolute differences were tested using a paired t-test (total IGF-I, IGFBP2, IGFBP-3, lycopene and SHBG) or Wilcoxon signed ranks test (free IGF-I, IGFBP-1, estradiol); relative differences were tested using a one-sample t-test (total and free IGF-I, IGFBP-2, IGFBP-3, lycopene and SHBG) or sign test (IGFBP-1 and estradiol).

Table 4.4. Mean serum concentration of IGF system components after lycopene intervention and after placebo, and within-individual cross-over difference between lycopene intervention and placebo, for the two study populations separately

	Breast cancer survivor population (*n* = 24)				Family history population (*n* = 36)			
	Concentration after lycopene	Concentration after placebo	Within-person difference absolute	Within-person difference relative (%)	Concentration after lycopene	Concentration after placebo	Within-person difference absolute	Within-person difference relative (%)
Total IGF-I (µg/L)	161.9 ± 49.0	153.0 ± 47.8	8.8	7.0 (-0.2 - 14.3)	182.9 ± 46.0	191.0 ± 48.3	- 8.1	- 3.3 (-7.6 - 1.0)
Free IGF-I (µg/L)[a]	0.55 (0.31-1.89)	0.57 (0.22-1.26)	0.0	6.3 (-3.5 - 16.1)	0.69 (0.26-1.75)	0.74 (0.31-2.38)	- 0.06[b]	- 7.6 (-14.6 - -0.6)[b]
IGFBP-1 (µg/L)[a]	41 (7 - 84)	42 (6 - 152)	- 2	-7.3 (-50.7 - 27.5)	36 (12 - 177)	33 (9 - 108)	- 1	- 2.4 (-46.9 - 240.4)
IGFBP-2 (µg/L)	309.9 ± 119.6	329.4 ± 119.6	- 19.5	-5.1 (-11.6 - 1.3)	262.8 ± 128.8	255.1 ± 110.9	7.7	2.4 (-4.7 - 9.5)
IGFBP-3 (mg/L)	2.60 ± 0.45	2.52 ± 0.46	0.08[b]	3.3 (0.7 - 6.0)[b]	2.50 ± 0.33	2.56 ± 0.40	- 0.05	- 1.3 (-4.4 - 1.9)

[a] Absolute concentrations and difference in free IGF-I and IGFBP-1, and relative difference in IGFBP-1 are not normally distributed, therefore median (and range) instead of mean and standard deviation are reported. Tests: paired samples t-test (total IGF-I, free IGF-I, IGFBP-2, and IGFBP-3) or Wilcoxon signed ranks test (IGFBP-1) for absolute differences, and one-sample t-test (total IGF-I, free IGF-I, IGFBP-2, and IGFBP-3) or sign test (IGFBP-1) for relative differences for IGFBP-1 the median and range of the relative difference is reported, as the relative difference is not normally distributed; [b] $p < 0.05$

familial risks of breast cancer, i.e., the 'breast cancer survivor population' and the 'family history population', which were recruited in two separate trials, we also exploratively analyzed the results of each group separately. The effects of lycopene supplementation statistically significantly differed between the two groups for total and free IGF-I and for IGFBP-3 (**Figure 4.3**).

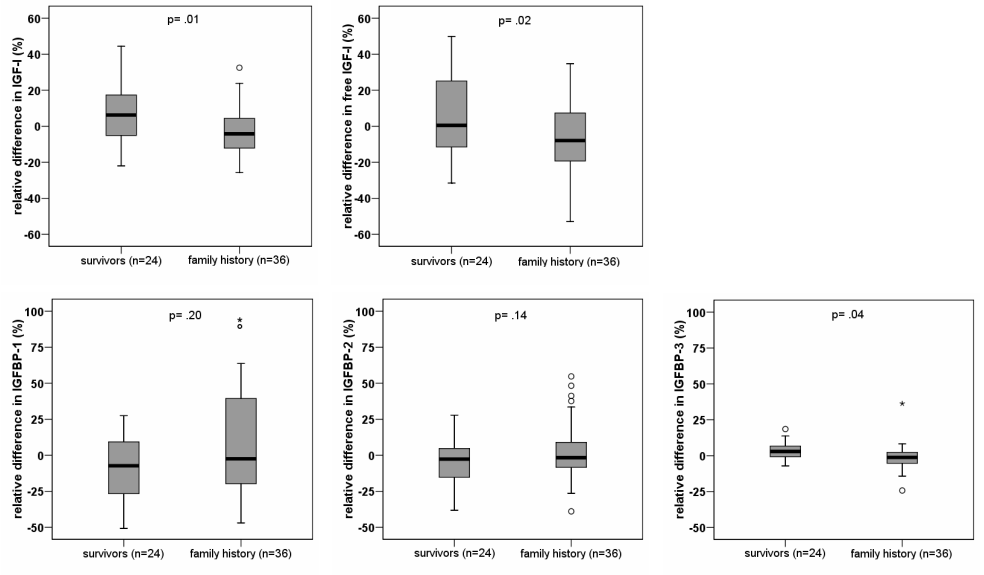


Figure 4.3. Distribution of within-individual differences between serum concentrations of IGF system components after lycopene as compared to after placebo, for the breast cancer survivor population and the family history population separetely. Boxplots indicating: median (horizontal bar), interquartile range (box, 25th to 75th percentile), and the range including outliers (° values between 1.5 and 3 box lengths from the 25th and 75th percentile, * values more than 3 box lengths from the 25th and 75th percentile). P-values for Wilcoxon signed ranks tests for differences between the breast cancer survivor population and the family history population.

Within the 'breast cancer survivor population' a marginal, statistically significant, increase of 3.3% (95%CI 0.7 to 6.0%) in IGFBP-3 concentration was observed, as well as non-significant increases in total and free IGF-I and decreases in IGFBP-1 and –2 (**Table 4.4**). In contrast, within the 'family history population' a significant decrease in free IGF-1 was observed, of –7.6% (95%CI –14.6 to –0.6%), which was accompanied by a non-significant decrease in total IGF-I and minor changes in the levels of IGFBPs.

Discussion

To our knowledge, this study is the first randomized trial to examine the effects of lycopene supplementation on IGF system components in premenopausal women at

increased risk of breast cancer, and the largest trial in humans thus far. This randomized placebo-controlled double-blind cross-over trial of two months of lycopene supplementation did not show an overall effect on serum IGF-I. However, lycopene effects were discordant between the two study populations showing beneficial effects in the high-risk healthy women but not in breast cancer survivors.

There were several strengths as well as limitations to our study. We used a cross-over design, which has the advantage of investigating the effect of lycopene as compared to placebo within individuals. By using this approach we eliminated between individual variation in circulating IGF(BP)-levels, resulting in high statistical power to detect differences between lycopene supplementation and treatment with placebo. On account of the randomization it is highly unlikely that any effects of lycopene supplementation on the IGF system are nullified by other factors. Indeed, relevant covariables, such as sex steroid hormonal status, energy and macronutrient intake, body weight, waist circumference, and physical activity levels of participants remained largely unchanged throughout the study period. We investigated the effect of lycopene supplementation in high risk populations for breast cancer that could potentially benefit most from this intervention, as high serum IGF-I levels have been repeatedly associated with an increased risk of breast cancer (1;2). Exclusion of participants who were considered non-compliant (n = 13), i.e., as based on <20% increase of serum lycopene level or >20% of the capsules returned, did not markedly influence the results (data not shown). Overall, we observed approximately a doubling in plasma lycopene concentrations, similar to the increase observed in other human intervention studies after several weeks of supplementation with 15-60 mg/d of lycopene from tomato oleoresin supplements (34-36). The dose and duration of lycopene supplementation are similar to other trials in which metabolic effects of lycopene were observed (37-39). In the LP-group, which received lycopene in the first period, the plasma lycopene concentrations returned to baseline levels after the wash-out period. Therefore, carry-over effects with respect to effects on serum IGF-system components are unlikely. Effects of lycopene supplementation on the IGF-system in the breast are unknown. Lycopene may be stored in breast adipose tissue (40), and exert local effects on the IGF-system. However, it was not feasible to collect breast tissue samples in the setting of this randomized, controlled trial.

Although it must be emphasized that we had limited power to detect intervention effects on secondary endpoints within the two study populations separately, our results suggest that the lycopene intervention lead to opposite results between these groups. The results in the 'family history population' were as hypothesized, i.e., decreased concentrations of total and free IGF-I. In contrast, increased concentrations of IGFBP-3, and total and free IGF-I were observed in the 'breast cancer survivor population'. Total serum IGF-I has been the most frequently studied, and relatively high concentrations are shown to be associated with an increased risk of breast cancer in premenopausal women (1;2). Free IGF-I, i.e., IGF-I not bound to any of its IGF binding proteins, is thought to reflect bioavailability of IGF-I to bind to the IGF receptor. However, binding of IGF-I to certain

binding proteins, i.e., IGFBP-1 and IGFBP-2, but not IGFBP-3, may actually facilitate transport of IGF-I out of the bloodstream to specific target tissues, thereby possibly also affecting IGF-I bioavailability (2). Only one prospective study thus far evaluated serum concentrations of free IGF-I and breast cancer risk and showed no association (41).

Several differences between the populations could underlie the differential lycopene effects observed. The most important difference is of course disease status. However, tumor cell effects and breast cancer treatment effects as possible explanations are unlikely, as malignancies in the 'breast cancer survivor population' were diagnosed more than 4 years before start of the intervention, patients had no local or distant metastases at diagnosis or during follow-up, and had not been treated with chemotherapy or hormonal therapies. Current oral contraceptive use also markedly differed between the two populations, i.e., no current use in the 'breast cancer survivor population' whereas almost half of the 'family history population' were current users. Sex steroid hormones are known to interact with the IGF system. However, the non-significant change in estradiol was similar in the two populations, and within the 'family history population' the lycopene effects did not differ between users and non-users of oral contraceptives (data not shown). Multivariate analysis showed that age was not a significant determinant of lycopene effects (data not shown).

More likely, familial or genetically determined baseline differences in the IGF system may explain the discordant results in the two populations. Concentrations of IGF system components after placebo were less favorable in the 'family history population', which is in accordance with the literature (12). Decreases in total or free IGF-I by lycopene intervention may be more easily achieved in individuals with relatively high baseline levels of IGF-I. In addition, specific (epi)genetic alterations like a BRCA1 mutation might have their own effects on the IGF system (42-44). With some caution, our results suggest that lycopene may have unwanted effects on the IGF system in breast cancer survivors, but beneficial effects in healthy women with a family history of breast cancer. However, we cannot exclude that the differences in effects of the lycopene intervention between the two study populations are due to chance.

So far, five other studies have investigated the effects of lycopene on the IGF system in humans. One small randomized clinical trial in men studied the effects of three weeks of lycopene supplementation (Lyc-o-Mato, 30 mg lycopene/day) before radical prostatectomy (27). Serum IGF-I levels were decreased by about 30% in both the intervention group (n = 13) and in the control group (n = 10). However, plasma lycopene levels did not significantly change in either group during this short intervention. In a similar design (n = 56) among patients undergoing colectomy a 25% decrease in serum IGF-I levels was observed after lycopene supplementation (45). In our own cross-over trial of patients at increased risk of colorectal cancer due to a family history or previous adenomas (n = 71) we found no significant change in serum IGF-I after lycopene supplementation (Vrieling et al. in press). However, we did observe significant increases in IGFBP-1 and IGFBP-2 which may affect IGF bioavailability. Riso et al. reported on a 26-

day Lyc-o-Mato tomato drink intervention (5.7 mg lycopene/day) in 20 healthy subjects and did find markedly increased levels of serum lycopene (28). No overall reduction in serum IGF-I was observed after the tomato drink intervention as compared to the placebo. When analyses were limited to subjects in whom the lycopene concentration increased by more than 25 μmol/L or 100% of basal levels (*n* = 11), a small but significant decrease (5.7%) in IGF-I levels was observed after the tomato drink intervention. In our study, limiting the analyses to responders defined in this way did not markedly affect the results (data not shown). In a small parallel study (*n* = 20) of synthetic lycopene supplementation (15 mg lycopene/day) in healthy male volunteers no statistically significant effects on serum IGF-I and IGFBP-3 were observed (29). Inconsistencies between these studies may be due to differences in the study populations, as well as differences in the source of lycopene used, i.e., Lyc-o-Mato capsules, Lyc-o-Mato tomato drink, synthetic lycopene. Effects of lycopene may also differ depending on whether it is administered as whole tomato, tomato extract, or pure lycopene.

In conclusion, our study in premenopausal women at increased breast cancer risk confirms the IGF-I lowering effects of lycopene suggested by animal experiments and previous (small) trials and observational studies in humans, but only in healthy women, not in breast cancer survivors. Genetic background or disease status may interfere with lycopene effects of the IGF system. As the IGF system is known to interact with sex steroid hormone pathways, any effects of dietary factors on the IGF system may also differ by gender and menopausal status.

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Lycopene supplementation elevates circulating insulin-like growth factor binding protein -1 and -2 concentrations in persons at greater risk of colorectal cancer

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Abstract

Background: Higher circulating insulin-like growth factor I (IGF-I) concentrations have been related to a greater risk of cancer. Lycopene intake is inversely associated with cancer risk, and experimental studies have shown that it may affect the IGF system, possibly through an effect on IGF-binding proteins (IGFBPs).

Objective: The objective of our study was to investigate the effect of an 8-wk supplementation with tomato-derived lycopene (30 mg/d) on serum concentrations of total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3.

Design: We conducted a randomized, placebo-controlled, double-blinded crossover study in 40 men and 31 postmenopausal women with a family history of colorectal cancer, a personal history of colorectal adenoma, or both.

Results: Lycopene supplementation significantly (*P = 0.01*) increased serum IGFBP-1 concentrations in women (median relative difference between serum IGFBP-1 concentrations after lycopene supplementation and after placebo, 21.7%). Serum IGFBP-2 concentrations were higher in both men and women after lycopene supplementation than after placebo, but to a lesser extent (mean relative difference 8.2%; 95% CI: 0.7%, 15.6% in men and 7.8%; 95% CI -5.0%, 20.6% in women). Total IGF-I, IGF-II, and IGFBP-3 concentrations were not significantly altered by lycopene supplementation.

Conclusions: This is the first study known to show that lycopene supplementation may increase circulating IGFBP-1 and IGFBP-2 concentrations. Because of high interindividual variations in IGFBP-1 and IGFBP-2 effects, these results should be confirmed in larger randomized intervention studies.

Introduction

A Western lifestyle is positively associated with cancer risk, partially through effects on insulin and the insulin-like growth factors (IGFs) (1). Both insulin and IGF-I can stimulate tumor growth by inducing proliferation and inhibiting apoptosis. IGF-binding proteins (IGFBPs) are considered to both inhibit and stimulate the interaction of IGF-I with the IGF-I receptor (2). Prospective epidemiologic studies indicate that relatively high circulating total IGF-I concentrations are associated with greater risks of prostate, premenopausal breast, and colorectal cancer, whereas both positive and negative associations have been reported for IGFBP-3 (3). In addition, a few reports suggest that higher IGF-II (4) and lower IGFBP-1 (5;6) and IGFBP-2 (5) concentrations are associated with a greater risk of colorectal cancer. Circulating concentrations of IGFs and various IGFBPs are known to be influenced by dietary habits and other lifestyle factors (7).

Lycopene, the major carotenoid in tomatoes and tomato products, may inhibit cancer cell proliferation by interfering with the IGF system. In vitro studies in mammary and prostate cancer cells found that lycopene reduced IGF-I receptor signaling by increasing the concentrations of (membrane-associated) IGFBPs (8-10). In ferrets, both low- and high-dose lycopene supplementation for 9 wk (equivalent to 15 and 60 mg/d, respectively, in humans) significantly increased plasma IGFBP-3 concentrations and significantly decreased lung cancer development, whereas plasma IGF-I concentrations did not change significantly (11). Several studies in mice and rats also showed a reduction in cancer risk after lycopene supplementation but did not investigate effects on the IGF system (12;13).

Habitual dietary intake of lycopene in humans (mean intake range: 0.6 – 10.9 mg/d) did not appear to significantly affect colorectal cancer risk in a recent pooled analysis of 11 cohort studies (14), whereas results for prostate (15) and premenopausal breast (16) cancer have remained inconsistent. In 3 of 6 cross-sectional studies (17-22), higher intakes of cooked or processed tomatoes or lycopene were associated with either lower IGF-I concentrations (18), higher IGFBP-3 concentrations (19), or a lower molar ratio of IGF-I to IGFBP-3 (20). In the other 3 studies, no such associations were found (17;21;22). However, habitual dietary lycopene intake is generally low and weakly correlated with blood lycopene concentrations (23). To accurately investigate whether lycopene can affect the IGF system in humans, supplementation studies are needed.

Therefore, we conducted a randomized, placebo-controlled trial to investigate the effect of a 2-mo supplementation with tomato-derived lycopene (30 mg/d) on serum concentrations of IGF-I and -II and IGFBP-1, -2, and -3 in men and women at greater risk of colorectal cancer. This population and other populations at greater risk of cancer could potentially benefit the most from this intervention.

Subjects and Methods

Study population

We selected men aged 40-75 y and postmenopausal women aged 50-75 y who have had colorectal adenoma or who had ≥1 first-degree family member with a history of colorectal cancer. Asymptomatic individuals scheduled to undergo a colonoscopy for screening purposes were selected from medical registries and pathology databases and were sent a letter inviting them to participate in the study. Exclusion criteria were a history of cancer, familial adenomatous polyposis syndrome, familial Li-Fraumeni syndrome, chronic inflammatory bowel disease, diabetes mellitus, acromegaly, significant liver or renal disease, (partial) bowel resection, nonremissive celiac disease, diverticulitis, other severe comorbidity, laxative abuse, or the use of food supplements containing lycopene. Participants were recruited between July 2003 and September 2005, and they were randomly assigned to the lycopene trial described here or to a trial of isoflavones that was described previously (24). In total, 146 men and 182 women were invited to participate in the present trial. Of 126 eligible men, 60 were willing to participate (48% response), of whom 42 were included in the present trial. Of 156 eligible women, 41 were willing to participate (26% response), of whom 34 were included in the present trial. Most of those who were not willing to participate indicated that they had no time to participate or had no interest in the study. The study was conducted in 4 hospitals in the Netherlands: the Antoni van Leeuwenhoek Hospital (Amsterdam), the Gelderse Vallei Hospital (Ede), the Slotervaart Hospital (Amsterdam), and the Sint Antonius Hospital (Nieuwegein).

We obtained written informed consent from all participants. The study protocol was approved by the medical ethics committees of all participating centers.

Design

We conducted a randomized, placebo-controlled, double-blinded crossover study. The total duration of the study was ≈6 mo; it consisted of two 8-wk intervention periods, separated by an 8-wk wash-out period. Subjects were allocated to receive lycopene capsules in the first and placebo capsules in the second intervention period or vice versa, according to a randomization scheme with permuted blocks. The lycopene capsules (Lyc-O-Mato; LycoRed Natural Product Industries, Beer-Sheva, Israel) contained an extract (oleoresin) derived from tomatoes that represented ≈15 mg total lycopene/capsule. Subjects were asked to take 2 capsules/d - 1 capsule with breakfast and 1 with dinner (total dose: 30 mg lycopene/d). Subjects were asked to maintain their habitual diet and lifestyle.

Data collection

At the start of the study, subjects filled in a general questionnaire about their smoking behavior, family history of cancer, and hormonal factors. They visited their respective hospitals at the beginning and end of both intervention periods. At each of the 4 visits, body weight and circumferences of waist and hip were measured. Dietary intake on the

day before each visit was assessed during an in-person interview by using a 24-h recall method. Most of the men (41 of the 42) and 5 of the 34 women had to undergo bowel preparation for colonoscopy (for screening purposes) on the day before the second visit. In these cases, the 24-h recall related to the second day before the visit. The methods of interviewing and coding of foods and portion sizes were standardized, and these procedures were performed by trained nutritionists and graduate students in nutrition. Energy and nutrient intakes were calculated by using the VBS food calculating system (BAS Nutrition Software, version 4.0.57; B-Ware Nutrition Software, Wageningen, Netherlands) based on the Dutch food composition table (25).

Habitual physical activity over the 2 mo preceding each visit was assessed by using the validated self-administered short questionnaire to assess health-enhancing physical activity (26). During both intervention periods, subjects kept a daily notebook in which they recorded information about their health, medicine use, smoking, and frequency of consumption of products rich in lycopene (ie, tomato products and specific fruit). Compliance was measured by a counting of returned capsules, self-reported supplement intake from daily notebooks, and measurement of serum lycopene concentrations at the beginning and end of both intervention periods.

Laboratory analyses

Fasting serum and EDTA-plasma samples were frozen and stored at -30 and -80 ^{o}C, respectively, until further analysis. Serum total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations were measured at the end of both intervention periods. Serum total IGF-I was measured by using an immunometric technique on the Immulite 1000 analyzer (Diagnostics Products Corporation, Los Angeles, CA). The sensitivity established in our laboratory was 12.0 μg/L; intraassay CVs were <4.0% at 45, 150, and 370 μg mean serum IGF-I/L; and interassay CVs were 7.0%, 6.5%, and 7.0% at 45, 150, and 370 μg mean serum IGF-I/L, respectively. Serum IGF-II concentrations were measured in C18 extracts (Sep-Pak Cartridges; Waters Corp, Milford, MA) of serum by using a radioimmunoassay, as described previously (27;28). The sensitivity established in our laboratory was 0.09 μg/L; the intraassay and interassay CVs were 6.7% and 8.8%, respectively, at 505 μg mean serum IGF-II/L. Serum IGFBP-1, IGFBP-2, and IGFBP-3 concentrations were measured by using specific radioimmunoassays. Relevant technical details were described previously (27;29;30). Assays for total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 were performed at the Department of Endocrine and Metabolic Diseases, University Medical Center Utrecht.

Plasma concentrations of lycopene were measured at all 4 time points by using HPLC according to the method described by Gueguen et al (31). Samples were kept in the dark and stored at -80 ^{o}C until HPLC analysis was conducted. HPLC separation was achieved on a glass column (10 cm x 3-mm internal diameter; Varian Chrompack, Palo Alto, CA) packed with Nucleosil C18 material (Machery-Nagel, Duren, Germany) with the use of a mobile phase consisting of methanol:acetonitrile:tetrahydrofuran (75:20:5; vol/vol/vol) delivered at a flow rate of 0.4 mL/min; detection was conducted at 472 nm. The sensitivity in our at

laboratory was established as 0.01 μmol/L.
Because the IGF-system may be influenced by changes in estradiol, sex hormone binding globulin (SHBG), and insulin concentrations, we also measured estradiol and SHBG concentrations in women and insulin concentrations in both men and women. The immunoassays were based on the electrochemiluminescence principle and were used on the E170 immunoanalyzer (Elecsys module; Roche Diagnostics, Mannheim, Germany). Lycopene, estradiol, and SHBG assays were performed at the Department of Clinical Chemistry, The Netherlands Cancer Institute.

Statistical analyses
The main variable of interest in our statistical analysis was the relative crossover difference (*see* equations below), expressed as a percentage relative to the concentration after placebo. The crossover differences in IGF-I, IGF-II, IGFBP-2, IGFBP-3, SHBG, and insulin (in women) were normally distributed. The mean crossover difference for each of these variables was calculated for both intervention groups and then pooled to adjust for period effects. We tested whether the pooled crossover difference significantly deviated from the null value by using a *t* test in men and women separately (2-sided $\alpha = 0.05$, df = 39, and df = 30, respectively) with the use of pooled SEM crossover differences (32) as calculated by the following equations:

Relative cross-over difference [Δ(IP) or Δ(PI)] =
[concentration after intervention (C_I) - concentration after placebo (C_P)]/C_P (*1*)

Pooled cross-over difference = ½ ($\text{mean}_{\Delta(IP)} + \text{mean}_{\Delta(PI)}$) (*2*)

Pooled variance (s^2) = $[(n_{IP} - 1)SD^2_{\Delta IP} + (n_{PI} - 1)SD^2_{\Delta PI}]/(n_{IP} + n_{PI} - 2)$ (*3*)

Standard error of the pooled cross-over difference = $\frac{1}{2}\sqrt{[(s^2/n_{IP}) + (s^2/n_{PI})]}$ (*4*)

where IP is isoflavones-placebo, PI is placebo-isoflavones, s^2 is pooled variance, *n* is the number of subjects in the specific group, and SD^2 is the SD of the specific group. Because of the skewed distribution of IGFBP-1, estradiol, and insulin (in men), the median crossover differences were compared with the null value by using a univariate sign test.
All statistical analyses were conducted based on the basis of the intention-to-treat principle, including all participants who were randomly assigned and who donated a blood sample on all 4 study visits, irrespective of compliance with the intervention protocol. As a secondary approach, per-protocol analyses were conducted, excluding all participants who were noncompliant - ie, who took <80% of capsules (according to the count of returned capsules), who had a <20% increase in serum lycopene concentration after lycopene intervention, or both.
Descriptive characteristics were computed for men and women separately for the 2 randomized groups. We calculated whether dietary and lifestyle factors known to influence the IGF system – ie, dietary intake of macronutrients, body weight, waist and hip circumferences, total physical activity score, dietary intake of products relatively rich in lycopene (tomato products and specific fruit) - were significantly different in the

lycopene period than in the placebo period. Statistical analyses were performed by using SPSS software (version 12.0; SPSS Inc, Chicago, IL).

Results

After randomization, 5 individuals dropped out of the trial (drop-out rate: 7%). Only one participant dropped out of the study during the lycopene intervention period, and the reason was nausea. This left 21 men and 14 women in the lycopene – placebo (L-P) group and 19 men and 17 women in the placebo – lycopene (P-L) group who completed the study protocol.

Men in the P-L group were older, somewhat heavier, and less likely to be current smokers and had a lower baseline energy intake than those in the L-P group (**Table 5.1**). Women in the L-P group did not differ significantly from women in the P-L group with respect to these general characteristics. The number of participants (men and women separately) with a family history of colorectal cancer, a personal history of colorectal adenoma, or both was equally distributed between the 2 groups. Hormonal factors in women, (ie, age at menopause, parity, and past hormone use) did not differ significantly between the 2 groups (data not shown).

Lycopene supplementation did not significantly affect serum total IGF-I and IGF-II concentrations in men or in women (**Table 5.2**). We observed a large interindividual variation in IGFBP-1 and IGFBP-2 responses to lycopene supplementation in both men and women (data not shown). Serum IGFBP-1 concentrations were not affected in men; however, IGFBP-1 was significantly higher in women after lycopene supplementation than after placebo (median relative difference between lycopene and placebo: 21.7%; P = 0.01). Serum IGFBP-2 was significantly higher in men after lycopene supplementation than after placebo (mean: 8.2%; 95% CI: 0.7%, 15.6%), and it was similarly but not significantly higher in women (mean: 7.8%; 95% CI: -5.0%, 20.6%). Serum IGFBP-3 concentrations were not significantly affected by lycopene supplementation. However, we observed a positive association between relative changes in lycopene concentrations and relative changes in serum IGFBP-3 (r = 0.46, $P < 0.01$) and IGF-I (r = 0.37, P = 0.08) in women. Estradiol concentrations in women did not differ significantly after lycopene supplementation (median: 2.0%; P = 0.47), whereas SHBG concentrations were significantly higher (mean: 7.8%; 95% CI: 1.7%, 14.0%).

Insulin concentrations did not differ significantly between the lycopene and the placebo intervention periods (Table 5.2). However, insulin concentrations in men were significantly lower after the first intervention period (when the colonoscopy was performed) than after the second intervention period (median: 30.0 and 45.4 pmol/L, respectively; $P \leq 0.001$), a difference that is also expressed in the longer fasting duration after the first intervention period (mean: 18 and 12 h, respectively; $P \leq 0.001$). In women, no significant differences in insulin concentrations were observed (mean: 53.5 and 55.3 pmol/L, respectively; P = 0.63). Because insulin concentrations are known to be inversely

Table 5.1. Characteristics at the first visit for the lycopene–placebo (L-P) and placebo–lycopene (P-L) supplementation groups of men and women

	Men		Women	
	L-P group (*n* = 21)	P-L group (*n* = 19)	L-P group (*n* = 14)	P-L group (*n* = 17)
Age (y)	54.9 ± 11.5[a]	61.3 ± 7.2	62.1 ± 6.1	60.0 ± 5.8
Weight (kg)	83 ± 13	86 ± 9	75 ± 10	75 ± 8
Height (cm)	178.6 ± 8.1	178.3 ± 6.5	166.6 ± 5.9	166.7 ± 4.4
BMI (kg/m^2)	25.9 ± 2.7	27.1 ± 2.9	27.1 ± 4.8	27.2 ± 2.9
Waist circumference (cm)	95.6 ± 7.5	101.5 ± 9.2	90.4 ± 12.4	90.6 ± 9.5
Hip circumference (cm)	103.7 ± 6.1	105.8 ± 5.2	108.8 ± 10.4	109.4 ± 6.1
Waist-to-hip ratio	0.92 ± 0.05	0.96 ± 0.05	0.83 ± 0.07	0.83 ± 0.06
Smoking [*n* (%)]				
Current	6 (29)	2 (11)	3 (21)	2 (12)
Never	6 (29)	5 (26)	4 (29)	8 (47)
Past	9 (43)	12 (63)	7 (50)	7 (41)
History of cancer or adenoma [*n* (%)]				
Family history of colorectal cancer	7 (33)	2 (11)	0 (0)	2 (12)
Personal history of adenoma	6 (29)	10 (53)	9 (64)	9 (53)
Both	8 (38)	7 (37)	5 (36)	6 (35)
Energy (kJ/d)	10241 ± 2949	7693 ± 2061	8331 ± 2264	7281 ± 2284
Protein (% of energy)	17 ± 5	18 ± 6	16 ± 4	16 ± 4
Fat (% of energy)	35 ± 8	34 ± 9	39 ± 11	36 ± 8
Carbohydrates (% of energy)	45 ± 10	40 ± 8	42 ± 10	45 ± 9
Alcohol (% of energy)	5 ± 7	9 ± 9	4 ± 5	4 ± 6

[a] x ± SD (all such values).

related to IGFBP-1 and, to a lesser extent, IGFBP-2 concentrations, we determined whether relative changes in insulin and the IGFBPs were correlated. We observed strong inverse correlations between relative insulin changes and relative changes in IGFBP-1 (r = -0.51, $P \leq 0.001$) and IGFBP-2 (r = -0.62, $P \leq 0.001$) in men but no significant correlations in women (r = -0.35, P = 0.054, and r = 0.001, P = 0.995, respectively).

According to both returned-capsule counts and recordings in the daily notes books, 94% of the participants were compliant (≥80% of capsules taken). Serum lycopene concentrations were significantly ($P < 0.001$) higher after lycopene intervention than at all other time points. A mean 259% increase from 0.17 ± 0.13 μmol/L at baseline to 0.61 ± 0.22 μmol/L after lycopene intervention was observed. Excluding of subjects who were noncompliant (n = 5) according to serum lycopene concentrations, returned-pill counts, or both did not materially change the results (data not shown). Body weight, waist and hip circumferences, total physical activity score, dietary macronutrient intake, and the number of days on which products rich in lycopene were consumed did not materially differ between the lycopene and the placebo intervention periods (data not shown).

Table 5.2. Circulating insulin-like growth factor (IGF) system component concentrations after lycopene and placebo treatment and the within-person crossover difference between the lycopene and the placebo treatment[1]

	Concentration		Within-person difference between treatments	
	After lycopene[a]	After placebo[a]	Absolute mean	Relative mean (%) (95%CI)
Men (*n* = 40)				
Total IGF-I (μg/L)	138.8 ± 40.8[b]	144.7 ± 46.0	-5.9	-2.2 (-7.6, 3.2)
Total IGF-II (μg/L)	533.3 ± 106.7	552.7 ± 130.9	-19.4	-1.2 (-6.4, 4.0)
IGFBP-1 (μg/L)[c]	37 (15, 49)	32 (19, 43)	-2	-9.5 (-40.0, 19.5)
IGFBP-2 (μg/L)	287.7 ± 150.8	276.4 ± 139.3	11.3	8.2 (0.7, 15.6)
IGFBP-3 (mg/L)	1.97 ± 0.28	2.01 ± 0.33	-0.03	-0.9 (-4.1, 2.2)
Insulin (pmol/L)[c]	32 (24, 63)	37 (30, 56)	-6.5	-9.5 (-37.0, 58.7)
Women (*n* = 31)				
Total IGF-I (μg/L)	118.2 ± 32.8	127.8 ± 52.7	-9.6	-3.2 (-9.5, 3.0)
Total IGF-II (μg/L)	549.1 ± 112.8	522.4 ± 81.3	26.7	5.4 (-0.9, 11.7)
IGFBP-1 (μg/L)[c]	33 (22, 55)	28 (16, 48)	5.0	21.7 (-1.0, 47.2)
IGFBP-2 (μg/L)	257.4 ± 119.9	254.7 ± 126.0	2.7	7.8 (-5.0, 20.6)
IGFBP-3 (mg/L)	2.25 ± 0.27	2.23 ± 0.34	0.01	1.7 (-2.4, 5.8)
Insulin (pmol/L)	53 ± 26	57 ± 34	-4.0	0.1 (-11.0, 11.2)

[a] IGFBP, IGF-binding protein; [b] Pooled x ± SD (all such values); [c] Data for IGFBP-1 and insulin in men and for IGFBP-1 in women were not normally distributed; values are medians; interquartile ranges in parentheses. P = 0.20, P = 0.27, and P = 0.01, respectively (sign test).

Discussion

In this randomized, placebo-controlled, double-blinded crossover study, lycopene supplementation at 30 mg/d for 2 mo did not significantly alter serum total IGF-I, IGF-II, and IGFBP-3 concentrations in men and women at greater risk of colorectal cancer. However, serum IGFBP-1 in women and serum IGFBP-2 in men were significantly higher after supplementation, which may result in less IGF-I bioavailability.

This is the first randomized trial investigating the effects of lycopene supplementation on the circulating IGF system and on IGFBP-1 and –2 in a population at greater risk of colorectal cancer. This population and other populations at greater risk of cancer could potentially benefit the most from this intervention. We used a crossover design, which has the important advantage that the results were not affected by the high interindividual variation in circulating IGF component concentrations. Moreover, small baseline differences in age, weight, energy intake, and smoking status between the men in the L-P and P-L groups are not likely to have affected our results. The dropout rate was very low (7%) and unrelated to supplement intake. Compliance, based on capsule counts and daily notebooks, was very high, and this was also reflected in strongly increased serum lycopene concentrations in 96% of the participants. Serum lycopene concentrations after lycopene

intervention and after baseline were within the range of those previously observed in other studies using 15–60 mg tomato oleoresin supplements/d (33-35). Blinding was confirmed by the fact that only 20% of the participants correctly guessed the period in which they received the lycopene supplementation. Dietary and lifestyle factors that are thought to influence circulating concentrations of IGFs and IGFBPs were did not differ significantly between the lycopene and the placebo intervention periods.

For practical reasons (ie, bowel preparation for colonoscopy), the duration of fasting prior to blood withdrawal was significantly longer in the male participants at the end of the first intervention period than at the end of the second period. As a consequence, at the end of the first intervention period, we found significantly lower serum concentrations of insulin. However, the period of fasting and, hence, the serum insulin concentrations were similar in women in both intervention periods. Circulating total IGF-I, IGF-II, and IGFBP-3 concentrations were previously found not to be influenced by fasting for up to 72 h (36;37). In contrast, fasting for shorter times readily leads to an increase in IGFBP-1 in the circulation, which is induced by a depressed insulin secretion (36;37). The magnitude of the fasting-induced rise in the concentration of IGFBP-1 in the serum is related to insulin sensitivity ((38) and may therefore vary among the subjects investigated. Therefore, it is difficult to draw definite conclusions with respect to the relative contribution of lycopene supplementation to the observed alterations in circulating IGFBP-1 concentrations in the male subjects. Of the female participants, only 5 underwent bowel preparation for colonoscopy. Exclusion of these women did not significantly change the IGFBP-1 results for the total group (24.4%, $P = 0.01$), but it did strengthen the IGFBP-2 results (13.0%; 95% CI: 0.0%, 26.0%). Thus, the increase in IGFBP-1 in women is likely to be the result of a direct lycopene effect and is unlikely to be mediated by a decrease in insulin concentrations, which have previously been inversely associated with lycopene concentrations (39;40). Although the effect of short-term fasting on IGFBP-2 is still controversial, we also observed a high interindividual variation in IGFBP-2 effects that were due to lycopene supplementation in both men and women. Therefore, some caution must be taken in the interpretation of these results.

Lycopene may inhibit cancer growth by various mechanisms, and increasing experimental evidence suggests that lycopene may affect the IGF system (8-11;41;42). However, evidence with respect to lycopene and the circulating IGF system in humans is sparse. To our knowledge, only 4 small human intervention studies investigating the effect of lycopene supplementation on circulating IGF components have been conducted thus far (43-46). Kucuk et al (43) observed in a parallel study of 26 prostate cancer patients that plasma IGF-I and IGFBP-3 decreased from baseline in the intervention group receiving Lyc-O-Mato lycopene capsules (30 mg lycopene/d) for 3 wk. However, plasma lycopene concentrations did not change during the study period, and similar decreases in IGF-I and IGFBP-3 were observed in the control group who received no supplementation. Riso et al (44) conducted a crossover study in 20 healthy young persons (8 M, 12 F) in which they compared the consumption of one Lyc-O-Mato drink/d (5.7 mg lycopene/d) for 26 d with

that of a placebo drink without lycopene. In that study, changes in lycopene concentrations were inversely correlated with those in serum IGF-I in the total group, whereas IGFBP-3 concentrations were not affected by lycopene supplementation. Serum IGF-I was reduced in subjects with a relatively high lycopene response of an increase of >0.25 μmol/L, or $\geq 100\%$ of the basal concentrations. Graydon et al (45) observed a positive association between changes in lycopene concentrations and IGFBP-3 concentrations in a parallel study within 10 healthy men receiving lycopene supplementation for 4 wk (15 mg/d). No overall differences in serum IGF-I and IGFBP-3 were found between those 10 men and 10 men in the placebo group. In a recent, larger parallel study of 56 colon cancer patients by Walfisch et al (46), a significant 25% reduction in serum IGF-I was observed in the intervention group receiving Lyc-O-Mato lycopene capsules (30 mg lycopene/d) for various durations (mean: 10 d; range: 2-49 d), whereas serum IGF-I in the placebo group did not change significantly. No changes in serum IGFBP-3 and IGF-II were observed.

In our study, which was of stronger design and had a larger sample size than did the studies discussed above, we did not observe any effects of lycopene on serum IGF-I and IGFBP-3 in the total group or in the high-lycopene responders as defined by Riso et al (44). Although we did observe a positive association between relative changes in serum lycopene concentrations and relative changes in circulating IGF-I and IGFBP-3 concentrations in the female participants, we could not confirm such an association in the male participants, and interpretation of the results is difficult. However, these results suggest that lycopene may increase IGFBP-1 and -2 concentrations.

In conclusion, lycopene supplementation did not influence serum total IGF-I and IGFBP-3 concentrations in our randomized, placebo-controlled, double-blinded crossover trial in a population at greater risk of colorectal cancer. However, lycopene supplementation may decrease IGF-I bioavailability by increasing IGFBP-1 and -2 concentrations. Thus, it may provide a means of ultimately reducing colorectal cancer risk and potentially the risks of other major cancers such as prostate and premenopausal breast cancer. However, interindividual variation in IGFBP-1 and -2 effects was high, possibly complicated by differences in fasting duration and, consequently, insulin concentrations. Therefore, results must be confirmed in larger randomized intervention studies with control for the duration of fasting.

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Isolated isoflavones do not affect the circulating insulin-like growth factor system in men at increased colorectal cancer risk

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Abstract

Epidemiological studies show that increased insulin-like growth factor (IGF)-I concentrations are related to increased colorectal cancer risk. A reduced colorectal cancer risk has been associated with isoflavones, which might affect the IGF-system because of their weak estrogenic activity. We conducted a randomized, placebo-controlled, double-blind crossover study to investigate the effect of an 8-wk isolated isoflavone supplementation (84 mg/d) on serum concentrations of total IGF-I, free IGF-I, total IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3. Additionally, we investigated whether IGF-system component differences were related to concentrations of the more potent estrogenic isoflavone metabolite, equol. Our study population consisted of 37 men with a family history of colorectal cancer or a personal history of colorectal adenomas. Isoflavone supplementation did not significantly affect serum total IGF-I concentrations (relative difference between serum total IGF-I concentrations after isoflavone supplementation and after placebo: -1.3%, 95%CI –8.6 to 6.0%). Neither free IGF-I, nor total IGF-II, IGFBP-1, IGFBP-2, or IGFBP-3 concentrations were significantly altered. Interestingly, the change in serum IGF-I concentrations after isoflavone supplementation was significantly negatively associated with serum equol concentrations (r = -0.49, p = 0.002). In conclusion, isolated isoflavones did not affect the circulating IGF-system in a male high-risk population for colorectal cancer. However, to our knowledge, this is the first study that suggests that isoflavones might have an IGF-I lowering effect in equol producers only. This underlines the importance of taking into account equol status in future isoflavone intervention studies.

Introduction

Insulin-like growth factors (IGF)[c] are involved in cell proliferation and apoptosis, and are important in both normal and tumor growth (1). Prospective epidemiological studies indicate that higher circulating concentrations of IGF-I are associated with increased colorectal cancer risk, whereas, for IGF binding protein (IGFBP)-3, inconsistent associations have been found (2). In addition, higher concentrations of IGF-II and reduced concentrations of IGFBP-1 and IGFBP-2 may be associated with increased colorectal cancer risk (3). Circulating concentrations of IGF-I and IGFBP are known to be modifiable by exogenous factors (e.g., dietary habits or other lifestyle factors) (4).

Estrogenic substances, such as oral estrogen replacement therapy (OERT), are associated with decreased colon cancer risk (5). Both OERT (6;7) and selective estrogen receptor modulators (SERM) (8-10) reduce serum IGF-I concentrations. Isoflavones are a class of phyto-estrogens mainly present in soy products and extensively studied for their potential anti-carcinogenic effects (11). Isoflavones structurally resemble estrogens and possess weak estrogenic activity (12). Therefore, these compounds may induce an estrogen-like reduction of IGF-I in the circulation.

In vitro and *in vivo* animal studies indicate that (soy) isoflavones may decrease serum IGF-I concentrations, whereas cross-sectional studies in humans did not find clear associations between soy isoflavone intake and serum IGF-I concentrations (4). Several randomized controlled supplementation studies of soy protein isolates containing isoflavones in humans have found either no effect (13-15) or an increase (16-20) in serum IGF-I concentrations by soy isoflavones. The inconsistency in these results may be explained by potential IGF-increasing effects of soy protein itself, which can mask a potentially IGF-lowering effect of isoflavones alone (4).

Isolated isoflavones can be derived from red clover, which has a different isoflavone composition, but may result in a similar isoflavone blood profile as compared to soy products (21). To our knowledge, red clover isoflavones have not been previously studied for their IGF effects in men or in relation to risk of colorectal cancer. Therefore, we investigated the effect of a 2-mo isolated red clover isoflavone supplementation (84 mg/d) on serum concentrations of IGF and various IGFBP in a male population at increased risk of colorectal cancer that could potentially benefit most from this intervention. Because the biological effectiveness of isoflavones may depend on the individual ability to biotransform isoflavone metabolites to the more potent estrogenic metabolite equol (22), we also evaluated whether effects of isoflavones on circulating IGF were related to the ability to produce equol.

Materials and Methods

Study population

We selected men aged 40–75 y with a personal history of colorectal adenomas or with at least one 1st-degree family member with a history of colorectal cancer. Asymptomatic men, scheduled to undergo a colonoscopy for screening purposes, were selected from the medical registries and pathology databases and were sent an invitation letter to participate in our study. Exclusion criteria were a history of cancer, familial adenomatous polyposis syndrome, familial Li Fraumeni syndrome, chronic inflammatory bowel disease, diabetes mellitus, acromegaly, significant liver or renal disease, (partial) bowel resection, non-remissive celiac disease, diverticulitis, laxative abuse, other severe comorbidity, and the use of food supplements containing isoflavones. Participants were recruited between July 2003 and January 2005. In total, 154 men were invited to participate in this trial. Of 128 eligible men, 40 were included in this trial and 15 were assigned to another trial (43% response). We obtained written informed consent from all participants.

The study was conducted in 4 hospitals in the Netherlands: the Antoni van Leeuwenhoek Hospital in Amsterdam, the Gelderse Vallei Hospital in Ede, the Slotervaart Hospital in Amsterdam, and the Sint Antonius Hospital in Nieuwegein. The study protocol was approved by the Medical-Ethical Committees of all participating centers.

Design

We conducted a randomized, placebo-controlled, double-blind crossover study. The total duration of the study was ~6 mo, consisting of two 8-wk intervention periods separated by an 8-wk wash-out period. Surveillance colonoscopies in study participants were always planned at the end of the 1st intervention period. Subjects were allocated to receive isoflavone tablets in the 1st and placebo tablets in the 2nd intervention period [isoflavones-placebo (IP group)] or vice versa [placebo-isoflavones (PI group)], according to a randomization scheme with permuted blocks. The isoflavone tablets (Promensil, Novogen) contained an isoflavone extract derived from red clover containing 42 mg of total isoflavones (25 mg biochanin, 8 mg formononetin, 4 mg genistein and 5 mg daidzein). Subjects were asked to take 2 tablets/d, one with breakfast and one with dinner (total dose, 84 mg/d). Subjects were asked to maintain their habitual diet and lifestyle.

Data collection

Subjects visited the hospital at the beginning and end of both intervention periods. At each visit, blood samples were drawn after an overnight fast, and weight, waist and hip circumference were measured. To assess habitual diet of the study population, a 24-h recall was conducted at each study visit. The method of interviewing and coding of foods and portion sizes was standardized and was performed by trained nutritionists and graduate students in nutrition. Energy and nutrient intakes were calculated using the VBS food calculating system (BAS Nutrition Software) based on the Dutch food composition table (23). Habitual physical activity over the 2 mo preceding each visit was assessed using

the validated self-administered Short Questionnaire to Assess Health-enhancing physical activity (SQUASH) (24). During both intervention periods, subjects kept a daily notebook in which they recorded information about their health, medicine use, smoking, and consumption frequency of products rich in isoflavones (i.e., soy products, legumes, and nuts). Compliance was measured by counting returned tablets, self-reported supplement intake from daily notebooks, and serum genistein concentrations at the beginning and end of both intervention periods.

Laboratory analyses

Fasting serum and EDTA-plasma samples were frozen and stored at –30°C until further analysis. All IGF-system components were measured at the end of both intervention periods. Serum total IGF-I was measured using an immunometric technique on the Advantage Chemiluminescense System (Nichols Institute Diagnostics). The sensitivity was 6.0 μg/L, intra-assay CV were 8.0% and 6.0% (*n* = 25) at 30 and 450 μg/L mean serum IGF-I, and interassay CV were 8.7%, 5.8% and 6.5% (*n* = 115) at 33, 174 and 445 μg/L mean serum IGF-I, respectively. Plasma free / IGFBP dissociable IGF-I was measured using a highly sensitive 2-site immunoradiometric free IGF-I kit (Diagnostic Systems Laboratories). The sensitivity was 0.2 μg/L, and the interassay CV was 12% at a mean plasma free IGF-I concentration of 1.6 μg/L. Serum IGF-II concentrations were determined in Sep-Pak C18 extracts of serum by RIA, as described previously (25;26). The sensitivity was 0.09 μg/L and intra- and interassay CV were 6.7% and 8.8% (*n* = 12) at 505 μg/L mean serum IGF-II, respectively. Serum IGFBP-1, IGFBP-2, and IGFBP-3 were determined by specific RIA. Relevant technical details were described previously (25;27;28). Total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 assays were performed in the same laboratory.

Serum genistein was measured at all four time points using reversed phase HPLC with UV detection according to methods adapted from Supko et al. (29) and Busby et al. (30). The sensitivity was 37 nmol/L, and the accuracy and precision were within 100 ± 15% and 15%, respectively.

Serum equol concentrations were measured in samples collected after isoflavone intervention. TR-FIA kits (Labmaster) were used as described previously (31) and fluorescence was measured with a Victor 2 model 1420 spectrofluorometer (Wallac). The sensitivity was 3.3 nmol/L and intra-assay CV was 12.3% at a mean equol concentration 134 nmol/L.

Statistical analyses

The parameter of interest in our statistical analysis was the relative crossover difference (see formulas below), expressed as a percentage relative to the concentration after placebo. The mean crossover difference for each IGF system component was calculated for both intervention groups (IP and PI) and then pooled over the 2 intervention groups, to adjust for period effects. We tested whether the pooled cross-over difference significantly deviates from null with a t-test (2-sided $\alpha = 0.05$, df = 37) using the pooled standard error

of the mean cross-over differences (32).

Cross-over difference (Δ) = concentration after intervention (C_I) - concentration after placebo (C_P);
Pooled cross-over difference = ½ ($mean_{\Delta IP}$ + $mean_{\Delta PI}$);
Standard error of the pooled cross-over difference = ½ $\sqrt{[(s^2/ n_{IP}) + (s^2/ n_{PI})]}$;
where $s^2 = [(n_{IP}-1)\ sd^2_{\Delta IP} + (n_{PI}-1)\ sd^2_{\Delta PI}\ ] / (n_{IP} + n_{PI} - 2)$

Descriptive characteristics were computed for both randomized groups separately. We calculated whether relevant changes occurred in dietary and lifestyle factors known to influence the IGF-system (i.e., dietary intake of macronutrients, weight, waist and hip circumference, total physical activity score, and dietary intake of products relatively rich in isoflavones) during the study period for both intervention groups separately. To evaluate whether the relative crossover differences correlated with serum genistein and equol concentrations after isoflavone supplementation, Spearman correlation coefficients were calculated. Logarithmic curve estimation was performed to obtain **Figure 1**.

Results

Forty men were included in this trial. After randomization, one individual was ineligible because of a history of prostate carcinoma. One participant was diagnosed with esophageal cancer in the 1st intervention period, and one participant dropped out in the 2nd intervention period because of bowel complaints (drop-out rate 5%). This resulted in 17 men in the IP group and 20 men in the PI group who finished the complete study protocol.

Men in the IP group were slightly heavier, had a greater BMI and waist circumference, and were more often non-smokers than those in the PI group (**Table 6.1**). The number of participants with a family history of colorectal cancer and/or a personal history of colorectal adenomas was equally distributed over the 2 groups. Isoflavone supplementation did not significantly affect serum total IGF-I concentrations (mean relative difference between concentration after isoflavones and after placebo: -1.3%, 95%CI –8.6 to 6.0%) (**Table 6.2**). Concentrations of free IGF-I, total IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 also did not differ after isoflavone supplementation, with mean differences ranging from –1.2 to 4.0% (Table 6.2).

The change in serum IGF-I concentration did not correlate with serum genistein concentrations (Spearman r = -0.12, P = 0.49). However, the change in serum IGF-I concentrations and serum equol concentrations after isoflavone intervention were negatively correlated (r = -0.49, P = 0.002) (**Figure 6.1**). Using a cut-off value of 83 nmol/L (22), 9 of 37 men (24%) were classified as equol producers and, for 8 of these 9 men, serum IGF-I decreased after isoflavone intervention compared with placebo (median –15.1%; range –27.2 to –4.1%). For free IGF-I, a similar but weaker association with equol was found (r = -0.37, P = 0.03). Correlations of equol with changes in the other IGF

parameters were in the same direction, but were not significant (*P* = 0.05-0.78; data not shown).

Table 6.1. General characteristics of the IP and PI groups of men[a]

	IP[b]	PI[b]
n	17	20
Age, *y*	59.9 ± 9.7	59.8 ± 7.2
Weight, *kg*	90.0 ± 14.3	83.3 ± 12.7
Height[c], *cm*	179.2 ± 9.9	178.3 ± 6.5
BMI, kg/m^2	27.9 ± 3.1	26.2 ± 3.0
Waist circumference[d], *cm*	105.3 ± 9.0	99.4 ± 9.9
Hip circumference, *cm*	106.7 ± 7.9	104.9 ± 7.1
Waist to hip ratio	0.99 ± 0.06	0.95 ± 0.06
Smoking, *n (%)*		
Yes	2 (12)	5 (25)
Never	4 (24)	2 (10)
Past	11 (65)	13 (65)
Family history of colorectal cancer and/or adenomas, *n (%)*		
Family history and adenomas	3 (18)	5 (25)
Family history only	4 (24)	5 (25)
Adenomas only	10 (59)	10 (50)
Food supplement use	3 (18)	3 (15)

[a] Values are means ± SD or n (%); [b] IP and PI indicate the treatment order; IP started with isoflavones, and PI with placebo; [c] Height of 2 persons in the PI-group was self-reported; [d] Waist circumference of 1 person in the IP-group was missing.

Table 6.2. Circulating IGF system component concentrations after placebo and isoflavone treatment and within-person crossover difference between isoflavone and placebo treatment

	Concentration after placebo[a]	Concentration after isoflavones[a]	Within-person difference	
			Absolute[b]	Relative[c]
Total IGF-I, *μg/L*	141.6 ± 57.5	138.1 ± 56.2	-3.5	-1.3 (-8.6 to 6.0)
Free IGF-I[d], *μg/L*	0.63 ± 0.34	0.59 ± 0.26	-0.04	-1.2 (-9.0 to 6.7)
Total IGF-II, *μg/L*	562.1 ± 177.6	572.4 ± 163.9	10.3	4.0 (-2.2 to 10.2)
IGFBP-1[d], *μg/L*	40.5 ± 23.0	37.9 ± 24.2	-2.6	1.6 (-15.8 to 19.1)
IGFBP-2, *μg/L*	306.0 ± 142.0	310.5 ± 153.5	4.5	3.4 (-4.1 to 10.8)
IGFBP-3, *mg/L*	2.31 ± 0.54	2.32 ± 0.53	0.01	1.0 (-2.9 to 4.9)

[a] Values are pooled means ± SD, *n* = 37; [b] Values are means, *n* = 37; [c] Values are means (95% CI), *n* = 37; [d] Two (free IGF-I) and one (IGFBP-1) subjects in the IP group were excluded because concentrations were below the detection limit.

Based on returned tablet counts and recordings in the daily record books, 95% of participants were compliant (≥80% of tablets taken). For 29 of 37 men (78%), mean serum genistein concentrations after isoflavone intervention (252 ± 252 nmol/L; range 46 to 1274

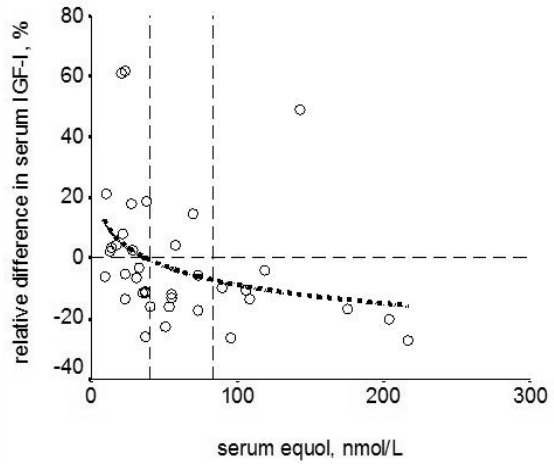

Figure 6.1. Serum equol concentrations vs. the relative difference (%) in serum IGF-I concentrations after isoflavone and after placebo supplementation in 37 men. Individuals with equol concentrations ≤40 nmol/L were previously defined as equol nonproducers (22), and individuals with equol concentrations ≥83 nmol/L as equol producers (dashed vertical lines).

nmol/L) had increased compared with all other time points (generally below detection limit of 37 nmol/L).
Body weight, waist and hip circumference, total physical activity score, dietary macronutrient intake, and the number of days that products rich in isoflavones were consumed did not materially differ for the isoflavone vs. the placebo intervention period (data not shown).

Discussion

In our randomized, placebo-controlled, double-blind crossover study, isolated isoflavone supplementation of 84 mg/d for 2 mo did not influence circulating IGF concentrations (total IGF-I, free IGF-I, and total IGF-II) and IGFBP (IGFBP-1, -2, and -3) in men at high risk of colorectal cancer. However, a relative decrease in serum total IGF-I concentrations after isoflavone intervention was observed with increasing individual serum equol concentrations, suggesting an IGF-I lowering effect of isoflavones in equol producers only.

To our knowledge, this is the first randomized controlled trial that correlates the effects of isolated isoflavones with both serum IGF-I and equol status in men. We used a crossover design, which has the important advantage of obtaining results unaffected by the high interindividual variation in circulating IGF-concentrations relative to the much lower intraindividual variation. Our study population consisted of men at high-risk for colorectal cancer who could potentially benefit most from this isoflavone intervention. The number

of recruited individuals for our study was more than sufficient, and the dropout rate was low (5%) and unrelated to supplement intake, which resulted in an adequately powered study. Compliance was high (≥95%) and genistein concentrations were increased in 78% of the participants. Blinding was confirmed by the fact that only 22% of the participants correctly guessed in which period they received the isoflavone supplements. Furthermore, factors thought to potentially influence circulating concentrations of IGF and IGFBP did not materially change during the study; therefore, changes in various parameters of the IGF-system could mainly be attributed to the isoflavone intervention.

To our knowledge, only 1 pilot cross-over study investigated the effect of an isolated red clover isoflavone supplementation on the IGF-system in humans. This study did not observe any effects on serum IGF-I and IGFBP-3 after 1 mo in both pre- and postmenopausal women (33), which is in line with our results in men. The 2-mo duration of isoflavone supplementation in our study was likely to be sufficient to affect the circulating IGF-system, insofar as human intervention studies on oral estrogens and SERM also observed a decrease in serum IGF-I after 2 mo (6;8). The differential effects on IGF for isoflavones compared with that of estrogens and SERM may be explained by their much weaker estrogenic activity (34). However, serum genistein concentrations in subjects with high soy consumption can far exceed those of endogenous estrogens, reaching levels sufficient to produce relevant biological effects (12).

The biological effects of isoflavones may also depend on the source and metabolism of isoflavones. In previous randomized controlled studies, the separate effects of isoflavones, soy protein, and calcium on the circulating IGF-system were difficult to disentangle (4). Therefore, we investigated a source of isolated isoflavones, using a red clover supplement. Red clover isoflavones mainly consist of biochanin A and formononetin, which are metabolized to genistein and daidzein, respectively. Soy mainly contains the glucoside isoflavones genistin and daidzin, which are also metabolized to genistein and daidzein in the intestines (34). Differences in composition between soy and red clover have been shown not to affect bioavailability within individuals (35). Plasma genistein and daidzein concentrations after red clover isoflavone supplementation are within the range of plasma concentrations in subjects who traditionally consume a high-isoflavone soy-based diet (50-800 μg/L) (21;34). The bioavailability of isoflavones however, may vary up to 10-fold between individuals (35). We also encountered large interindividual differences in serum genistein concentrations after isoflavone intervention. However, the relative difference in serum IGF-I concentrations after isoflavones and placebo did not correlate with serum genistein concentrations.
Recently, it has been hypothesized that only equol producers may benefit from isoflavone consumption (22). Approximately 30-50% of individuals are able to convert daidzein, one of the main isoflavone metabolites, to the more potent estrogenic isoflavone equol (36). Mice and rats metabolize the majority of daidzein to equol (22), and their serum IGF-I

concentrations decreased with physiological dietary intakes of soy or genistein (37;38). Accordingly, the majority of equol producers in our study had decreased IGF-I concentrations after consuming isoflavones compared with placebo. IGF-I concentrations after placebo did not differ between equol producers and nonproducers, suggesting that equol production, rather than host factors associated with equol production, may be important with respect to IGF modulation (36).

In conclusion, isolated isoflavones did not influence serum IGF-I concentrations in our adequately powered, randomized, placebo-controlled, double-blind crossover trial in a male population at increased risk of colorectal cancer. These results suggest that the increased serum IGF-I concentrations observed in previous studies investigating soy food or soy protein supplementation are most likely due to soy protein, and not to isoflavones. Although based on small numbers of equol producers, our data suggest that isoflavones might have an IGF-lowering effect in equol producers only. Therefore, it is important that our results will be confirmed in larger human intervention studies among men and women using isolated isoflavones and giving consideration to equol status.

Acknowledgements

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No effect of red clover-derived isoflavone intervention on Insulin-like Growth Factor (IGF) system; a randomized cross-over trial in women

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To be submitted

Abstract

Context: Increased insulin-like growth factor (IGF)-I and IGF-II concentrations are related to increased colorectal cancer risk. Isoflavones have been associated with reduced colorectal cancer risk, and may affect the IGF-system because of their weak estrogenic activity.

Objective: The aim of the study was to investigate the effect of isolated isoflavones on serum concentrations of IGF-system components.

Design: Randomized, placebo-controlled, double-blinded, cross-over trial.

Setting: Four hospitals in the Netherlands.

Participants: Thirty-four postmenopausal women with a family history of colorectal cancer or a personal history of colorectal adenomas.

Intervention: Eight-week, red clover-derived isoflavone supplementation (84 mg/day) and placebo, separated by an eight-week wash-out period.

Main Outcome Measures: Serum IGF-I concentration was the primary study endpoint. Serum IGF-II and IGF binding proteins (IGFBP-1, -2, and -3) were also assessed. In a parallel analysis mRNA expression of IGF-I, IGF-II, IGFBP-3 and IGF-IR in normal colorectal tissue biopsies was evaluated.

Results: Isoflavone supplementation did not significantly affect serum concentrations of total IGF-I (mean relative within-person difference; IGF-I, -2.0%, 95%CI –8.0 to 3.9%). IGF-II and IGFBPs were also not significantly altered after isoflavone supplementation. Colorectal tissue mRNA expression of IGF-system components did not significantly differ between individuals on isoflavone supplementation and individuals on placebo.

Conclusions: The results of our trial, supported by qualitative review of isoflavone trials published to date, suggest that isoflavones do not significantly affect circulating levels of IGF-system components. Increased levels of IGF-I observed in some trials are likely due to simultaneous protein supplementation.

Introduction

Evidence is accumulating for a protective effect of estrogenic substances against colorectal cancer (1). In line with these observations, the incidence of colorectal cancer is consistently lower in women than in men. Sex hormone replacement therapy in postmenopausal women has been shown to decrease colorectal cancer risk as well as development of colorectal adenomas (2-4). Both sex hormone replacement therapy (5;6) and selective estrogen receptor modulators, e.g. tamoxifen (7;8), have been shown to reduce serum insulin-like growth factor (IGF) -I concentrations.

IGF-I and IGF-II are involved in cell proliferation and apoptosis, and are important in both normal and tumor growth (9). Prospective epidemiological studies have shown that relatively high circulating concentrations of IGF-I and IGF-II are associated with increased colorectal cancer risk (10). IGF binding protein (IGFBP) -3, which binds 90% of circulating IGF-I in humans, is not related to risk of colorectal cancer (10;11). However, relatively low concentrations of IGFBP-1 and IGFBP-2, compounds also known to affect IGF bioavailability, may be associated with increased colorectal cancer risk (12;13). Experimental evidence from mouse models has supported the rationale for cancer prevention through lowering the circulating levels of (bioavailable) IGF-I (14).

Lower incidence of several cancers including colorectal cancer in Asian countries has been attributed to substantially higher consumption of soy foods in these countries (15;16). Isoflavones, the main bioactive substances in soy, are a class of phytoestrogens that structurally resemble estrogens and also possess weak estrogenic activity (17). Isoflavones have been hypothesized to influence colorectal cancer risk, although results are inconclusive (18). *In vitro* and *in vivo* animal studies have shown that (soy) isoflavones may decrease (circulating) IGF-I concentrations (19). Evidence from intervention studies in humans however, is conflicting, possibly due to opposite effects of soy protein and soy isoflavones on serum total IGF-I.

We hypothesize that isolated isoflavones, through their estrogenic properties, may induce a reduction of (bioavailable) IGF-I in the circulation in postmenopausal women. Such endocrine changes may be accompanied by changes in the expression of IGF-system components in normal colorectal tissue. In this randomized, controlled, cross-over trial we investigated the effect of isolated, red clover-derived isoflavone supplementation (84 mg/day) for two months on serum concentrations of IGF-I, IGF-II, and IGFBP-1, -2, and -3, as well as tissue mRNA expression of IGF-system components, in postmenopausal women at increased risk of colorectal cancer that could potentially benefit most from this intervention.

Materials and Methods

Study population

We selected women aged 50 to 75 years with a personal history of colorectal adenomas or at least one first-degree family member with a history of colorectal cancer. All women were postmenopausal, i.e., no menstrual cycles in the past 12 months, and in case of hysterectomy postmenopausal status was confirmed based on serum FSH levels. Asymptomatic women scheduled to undergo a colonoscopy for screening purposes were selected from the medical registries and pathology databases, and were sent an invitation letter for participation in our study. Exclusion criteria were a history of cancer, familial adenomatous polyposis syndrome, familial Li Fraumeni syndrome, chronic inflammatory bowel disease, diabetes mellitus, acromegaly, significant liver or renal disease, (partial) bowel resection, non-remissive celiac disease, diverticulitis, other severe comorbidity, laxative abuse, and the use of food supplements containing isoflavones.

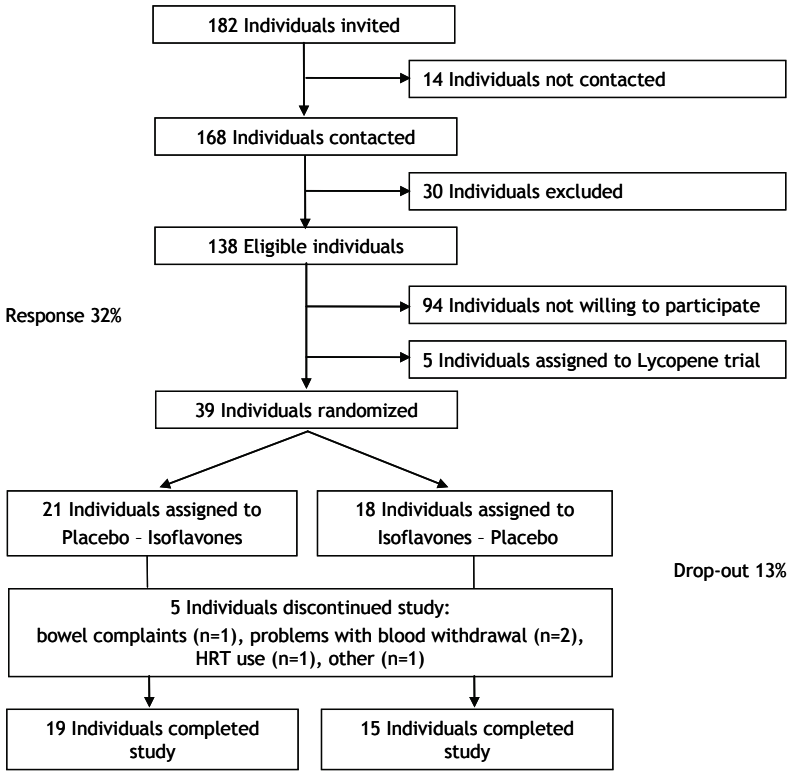
Participants were recruited between November 2003 and December 2005, and were initially randomized to one of two separate intervention trials. To increase enrollment, after one year randomization was restricted to the intervention trial currently described. In total, 182 women were invited to participate in this trial (**Figure 7.1A**). Of 138 eligible women, 44 were willing to participate of whom five women were assigned to the other trial and 39 were included in the present trial (32% response). We obtained written informed consent from all participants.

The study was conducted in four hospitals in the Netherlands: the Antoni van Leeuwenhoek hospital in Amsterdam, the Gelderse Vallei hospital in Ede, the Slotervaart hospital in Amsterdam, and the Sint Antonius hospital in Nieuwegein. The study protocol was approved by the Medical-Ethical Committees of all participating centers.

Design

We conducted a randomized, placebo-controlled, double-blinded cross-over study (**Figure 7.1B**). The total duration of the study was approximately six months, consisting of two eight-week intervention periods, separated by an eight-week wash-out period. Surveillance colonoscopies were always planned at the end of the first intervention period. Subjects were allocated to receive isoflavone tablets in the first and placebo tablets in the second intervention period [isoflavones-placebo (IP group)] or vice versa [placebo-isoflavones (PI group)], according to a randomization scheme with permuted blocks. The isoflavone tablets (Promensil, Novogen) contained an isoflavone extract derived from red clover containing 42 mg of total isoflavones (25 mg biochanin, 8 mg formononetin, 4 mg genistein and 5 mg daidzein). Subjects were asked to take two tablets per day, one with breakfast and one with dinner (total dose: 84 mg isoflavones/day). Subjects were asked to maintain their habitual lifestyle and diet, including non-isoflavone supplement use.

A



B

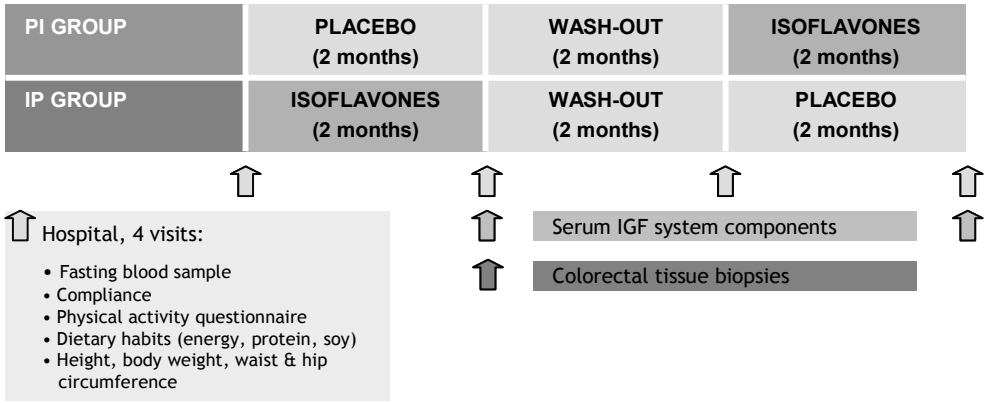


Figure 7.1. Flow chart of study recruitment and follow-up (A), and study scheme of cross-over design (B)

Data and sample collection

Study procedures and data collection were identical to a previous study described elsewhere (20). In summary, subjects visited the hospital at the beginning and end of both intervention periods, when body weight, and waist and hip circumference were measured. Dietary intake on the day preceding the visit was assessed using a 24-hour recall, according to a standard protocol for interviewing and coding. Habitual physical activity over the two months preceding each visit was assessed using a validated self-administered short questionnaire. Fasting blood samples were obtained at all four time points. Fasting serum and EDTA-plasma samples were frozen and stored at –30°C until further analysis.

A surveillance colonoscopy was scheduled at the second visit, i.e., at the end of the first intervention period, after whole-gut lavage with 4 L of macrogol (Klean-Prep (Norgine BV, Amsterdam, the Netherlands) or Coloforte (Ipsen Farmaceutica BV, Hoofddorp, the Netherlands)). Biopsies from macroscopically normal mucosa were collected from the ascending colon and the rectum, and were immediately snap-frozen in liquid nitrogen and stored at –70°C until further analysis.

During both intervention periods subjects kept a daily notebook in which they recorded information about their health, medicine use, smoking, study tablets taken, and consumption of foods rich in isoflavones. Compliance was measured by evaluating participants' own records and by counting the number of returned tablets.

Serum analyses

Serum total IGF-I, total IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 concentrations were measured at the end of both intervention periods. Serum total IGF-I was measured using an immunometric technique on the Immulite 1000 analyzer (Diagnostics Products Corporation, Los Angeles, USA). The sensitivity established in our laboratory was 12.0 μg/L, intra-assay CVs were <4.0% at 45, 150 and 370 μg/L mean serum IGF-I, and inter-assay CVs were 7.0%, 6.5% and 7.0% at 45, 150 and 370 μg/L mean serum IGF-I, respectively. Serum IGF-II concentrations were determined in Sep-Pak C18 extracts of serum by RIA, and serum IGFBP-1, IGFBP-2, and IGFBP-3 were determined by specific RIAs. All assays were performed in the same laboratory. Further details have been described previously (20).

Since the IGF-system may be influenced by changes in estradiol and sex hormone binding globulin (SHBG), we also determined estradiol and SHBG concentrations. The immunoassays were based on the electrochemiluminescence principle, and were used on the E170 (Elecsys module) immunoanalyzer (Roche Diagnostics, Mannheim, Germany).

Quantitative PCR

Total RNA was extracted from the tissue samples using RNAzolB (Campro Scientific). Total RNA (2.5 μg) was reverse-transcribed to generate first strand cDNA (total volume 50 μl) using random hexamers and Superscript II. The RT reaction was performed at 42°C for 60 min, followed by heating at 95°C for 2 min. cDNA was diluted 1:1 with RNAse free water

before real-time RT-PCR.

Quantitative PCR was used to assess the levels of mRNA expression of IGF-I, IGF-II, IGFBP-3, and IGF-IR in tissue samples obtained from the ascending colon (n=34), and rectum (n=34). Primers and probes for these reactions were designed using Primer Express software (Applied Biosystems, PE) (**Table 7.1**). Primers were chosen in two adjacent exons, and the fluorescent-labeled probes were selected to partially encompass both exons, to avoid DNA contamination and amplification of the homologous insulin and insulin receptor genes. PCRs were carried out using the 7500 Fast Real-Time PCR System (Applied Biosystems) according to manufacturer's instructions (50 cycles). The content of IGF-I, IGF-II, IGFBP-3, and IGF-IR transcripts was normalized to the content of the 'housekeeping gene' ß-actin. A second 'housekeeping gene' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed to evaluate the validity of normalization to ß-actin content. As the quantity of both genes was highly correlated (Spearman rank correlation coefficient: $r = 0.91$; $p < 0.001$), all results were normalized to ß-actin (GAPDH data not shown). For ß-actin and GAPDH, standard PCR primer and probe mixtures (Applied Biosystems) were used under the same conditions as described above. Standard curves were generated using serially diluted solutions of cDNA from pooled amplified RNA from 82 breast tumors. All PCR assays were conducted in duplicate for each sample.

Table 7.1. Primers and probes for real-time PCR

Gene	Exon	GenBank accession no.	Primer / probe[a]	Sequence
IGF-I	1,2	NM_000618	F	5'-AGCAGTCTTCCAACCCAATTATTTA-3'
			R	5'-AGATGCGAGGAGGACATGGT-3'
			Probe	5'-TCTTCACCTTCAAGAAATCACAAAAGCAGCA-3'
IGF-II	8,9	NM_000612	F	5'-CCGTGCTTCCGGACAACTT-3'
			R	5'-GGACTGCTTCCAGGTGTCATATT-3'
			Probe	5'-CCCAGATACCCCGTGGGCAAGTTCT-3'
IGFBP-3	2,3	BC018962	F	5'-AGAGCACAGATACCCAGAACTTCTC-3'
			R	5'-ATTGAGGAACTTCAGGTGATTCAGT-3'
			Probe	5'-CATTTCTCTACGGCAGGGACCATATTCTGTCT-3'
IGF-IR	8,9	X04434	F	5'-AAGGCTGTGACCCTCACCAT-3'
			R	5'-CGATGCTGAAAGAACGTCCAA-3'
			Probe	5'-TTCGCACCAATGCTTCAGTTCCTTCC-3'

[a] F = forward primer, R = reverse primer.

Statistical analyses

The main parameter of interest in our statistical analysis was the relative cross-over difference in total IGF-I (see formulas below), expressed as a percentage relative to the concentration after placebo treatment. Relative cross-over differences in serum concentrations of IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 were secondary endpoints. As the

sample size of the two randomized groups differed (i.e., PI-group, *n* = 19; IP-group, *n* = 15), the mean crossover difference for each IGF system component was calculated for both randomized groups and then pooled over the two groups, to adjust for period effects. We tested whether the pooled crossover difference significantly deviated from null with a t-test using the standard error of the pooled crossover differences (21). As IGFBP-1 and estradiol were not normally distributed, we tested whether the median cross-over differences significantly deviated from null using a sign test. Absolute differences between serum concentrations after isoflavones and after placebo were tested using paired t-tests (total IGF-I, IGFBP-2, IGFBP-3, SHBG) or Wilcoxon signed ranks tests (IGFBP-1, estradiol).

Cross-over difference (Δ) = concentration after intervention (C_I) - concentration after placebo (C_P)
Pooled cross-over difference = ½ ($mean_{\Delta IP}$ + $mean_{\Delta PI}$)
Standard error of the pooled cross-over difference = ½ $\sqrt{[(s^2/\ n_{IP})+ (s^2/\ n_{PI})]}$
where $s^2 = [(n_{IP}-1)\ sd^2_{\Delta IP} + (n_{PI}-1)\ sd^2_{\Delta PI}] / (n_{IP} + n_{PI} - 2)$

Differences in mRNA expression of the IGF-I, IGF-II, IGF-IR, and IGFBP-3 genes in normal colorectal tissue biopsies taken after the first intervention period were compared between individuals who received isoflavones intervention (IP-group) and individuals on placebo (PI-group). Natural log transformed data were used to normalize the data, and two-sample t-tests (IGF-I, IGF-II, IGFBP-3) or nonparametric Mann-Whitney test (IGF-IR) were performed.
Descriptive characteristics were computed for both randomized groups separately. We calculated whether relevant changes occurred in dietary and lifestyle factors known to influence the IGF system, i.e., dietary intake of macronutrients, body weight, waist and hip circumference, total physical activity score, dietary intake of products relatively rich in isoflavones, during the study period for both randomized groups separately.
P-values were determined by two-sided tests, and differences were considered to be statistically significant at *p*-values lower than 0.05. Statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Thirty-four women finished the complete study protocol [*n* = 19 on placebo – isoflavones (PI-group); *n* = 15 on isoflavones – placebo (IP-group)] (Figure 1A). Both groups were very similar with respect to age (**Table 7.2**). However, the PI-group was slightly more overweight and consisted of more never smokers. The number of participants with a family history of colorectal cancer and/or a personal history of colorectal adenomas was equally distributed over the two groups. Hormonal factors (i.e., age at menopause, parity, past use of hormone replacement therapy) also did not markedly differ between the two groups.

Table 7.2. General characteristics of the study population

	PI-group (*n* = 19)	IP-group (*n* = 15)
Age (years, mean ± SD)	58.9 ± 7.2	59.3 ± 4.3
Weight (kg, mean ± SD)	73.2 ± 12.9	71.4 ± 9.1
BMI (kg/m^2, mean ± SD)	26.7 ± 4.3	25.6 ± 3.2
Waist circumference (cm, mean ± SD)	90.3 ± 11.1	87.2 ± 9.9
Smoking (n (%))		
Yes	3 (16%)	5 (33%)
Past	8 (42%)	9 (60%)
Never	8 (42%)	1 (7%)
Colorectal cancer risk factor (n (%))		
Family history and adenomas	3 (16%)	5 (33%)
Family history only	7 (37%)	3 (20%)
Adenomas only	9 (47%)	7 (47%)
Age at menopause (years, mean ± SD)	47.9 ± 3.5	49.7 ± 5.3
Parity (n (%))		
Yes	13 (68%)	12 (80%)
No	6 (32%)	3 (20%)
Hormone replacement therapy[a] (n (%))		
Never	10 (56%)	11 (73%)
Past	8 (44%)	4 (27%)

[a] Percentage over valid values (missing, *n* = 1).

Isoflavone supplementation did not significantly affect serum total IGF-I and IGF-II concentrations (mean relative difference between isoflavones and placebo; IGF-I –2.0%, 95%CI –8.0 to 3.9%; IGF-II –1.7%, 95%CI –6.3 to 2.8%) (**Table 7.3**). Additionally, the mean relative differences in IGFBP-1, IGFBP-2 and IGFBP-3 between isoflavones and placebo did not significantly deviate from zero. However, substantial inter-individual variation in IGFBP-1 changes was observed, ranging from reductions of -50% to increases of up to 500%. Inter-individual variation in IGFBP-2 changes was smaller but still substantial. Serum estradiol and sex hormone binding globulin (SHBG) were also not significantly altered by the isoflavone intervention.

In a parallel analysis we studied tissue mRNA expression of the IGF-I, IGF-II, IGFBP-3, and IGF-IR genes measured in normal colorectal tissue biopsies taken after the first intervention period. No statistically significant differences were observed in mRNA levels of these genes between individuals on the isoflavones intervention and individuals on placebo, neither in the ascendening colon nor in the rectum (**Table 7.4**).

Based on both returned tablet counts and recordings in the daily note books, 91% of participants (*n* = 31) was compliant (≥80% of tablets taken) during both the isoflavone and the placebo intervention period. Excluding participants who were not compliant (*n* = 3) did not markedly affect the results (data not shown). Body weight, waist and hip circumference, total physical activity score, dietary macronutrient intake, and the number of days on which products rich in isoflavones were consumed did not materially

differ for the isoflavone versus the placebo intervention period (data not shown).

Table 7.3. Difference in serum concentration of IGF-system components after isoflavones intervention and after placebo

	Concentration after isoflavones[a]	Concentration after placebo[a]	Within-person difference		
			Absolute[b]	Relative[c]	
Total IGF-I (µg/L)	121 ± 45	123 ± 39	-3	-2.0	(-8.0 – 3.9)
Total IGF-II (µg/L)	546 ± 92	559 ± 98	-16	-1.7	(-6.3 – 2.8)
IGFBP-1 (µg/L)[d]	45 (2 – 206)	42 (2 – 121)	-2	-6.2	(-55.3 – 505.9)
IGFBP-2 (µg/L)	234 ± 137	223 ± 108	19	11.0	(-0.1 – 22.1)
IGFBP-3 (mg/L)	1.96 ± 0.30	2.01 ± 0.30	-0.04	-1.8	(-4.5 – 0.9)
Estradiol (pmol/L)[d]	42 (22 – 155)	42 (22 – 95)	1	2.7	(-53.3 – 344.2)
SHBG (nmol/L)	47 ± 17	47 ± 18	0	2.0	(-3.6 – 7.6)

[a] Values are pooled means ± SD, *n* = 34; [b] Values are means, *n* = 34; [c] Values are means (95% CI), *n* = 34; [d] Median and range, as IGFBP-1 and estradiol levels were not normally distributed.

Table 7.4. Normal colorectal tissue mRNA expression of IGF system components after isoflavones intervention or placebo (in arbitrary units)

	Placebo (*n* = 19)			Isoflavones (*n* = 15)			
	Mean ± sd	Median	Range	Mean ± sd	Median	Range	*P*-value[a]
			Ascending colon				
IGF-I	0.32 ± 0.20	0.33	0.09 – 0.84	0.40 ± 0.24	0.35	0.17 – 1.03	0.23
IGF-II	0.16 ± 0.11	0.14	0.04 – 0.45	0.14 ± 0.08	0.14	0.03 – 0.34	0.98
IGFBP-3	0.29 ± 0.17	0.25	0.04 – 0.66	0.29 ± 0.24	0.18	0.08 – 1.02	0.99
IGF-IR	2.58 ± 1.59	2.42	0.00 – 5.54	3.25 ± 2.58	2.71	0.00 – 10.91	0.66
			Rectum				
IGF-I	0.80± 0.46	0.85	0.19 – 1.90	1.04 ± 0.94	0.73	0.17 – 3.14	0.35
IGF-II	0.26 ± 0.11	0.25	0.05 – 0.45	0.30 ± 0.24	0.23	0.07 – 1.09	0.56
IGFBP-3	1.14 ± 0.57	1.10	0.18 – 2.28	1.25 ± 0.86	1.02	0.35 – 3.44	0.65
IGF-IR	7.10 ± 2.25	6.36	4.23 – 12.78	6.07 ± 2.28	6.80	2.98 – 11.08	0.47

[a] *P*-value based on independent t-test of natural log transformed IGF-I, IGF-II, and IGFBP-3, and Mann-Whitney test for IGF-IR.

Discussion

In our randomized, placebo-controlled, double-blinded, cross-over trial, red-clover derived isolated isoflavone supplementation of 84 mg/day for two months did neither influence circulating IGF-I and IGF-II concentrations nor IGF binding proteins (IGFBP-1, -2, and -3) in postmenopausal women at increased risk of colorectal cancer. In addition, colorectal tissue mRNA expression of IGF-I, IGF-II, IGF-IR, and IGFBP-3 did not differ between individuals on isoflavones and individuals on placebo.

This is the first randomized controlled trial investigating the effects of isolated isoflavones

on circulating IGF-system components in postmenopausal women at increased colorectal cancer risk. Populations at increased cancer risk, such as our study population, are likely to benefit most from interventions aimed at lowering circulating IGF-I levels and subsequently suppressing IGF-IR signaling. We used a cross-over design, which has the important advantage that our results have not been affected by the high between-individual variation in circulating IGF-I concentrations relative to the much lower within-individual variation.

The number of participants for our study was in concordance with the a priori design (i.e., $n \geq 26$). The drop-out rate, while 20% had been anticipated, was 13% and unrelated to supplement intake. This resulted in an adequately powered trial to evaluate isoflavone effects (i.e., 10% decrease) on serum IGF-I levels. Compliance, based on tablet counts and daily notebooks, was high (91%). In an identical trial in men with comparable compliance, a strong increase in serum genistein levels was observed in the majority of participants after isoflavone intervention (20). Furthermore, we also assessed factors which are thought to potentially influence circulating concentrations of IGFs and IGFBPs (e.g. total energy and protein intake, intake of products rich in isoflavones and lycopene, body weight, and physical activity). Since these factors did not materially change during the study, changes in various parameters of the IGF-system would have been attributable to the isoflavone intervention. The two-month duration of isoflavone supplementation in our study is likely to be sufficient to affect circulating IGF-system components, since human intervention studies on oral estrogens and SERMs also observed a decrease in serum IGF-I after two months (5;7).

To date 13 intervention studies in humans have evaluated the effect of isoflavone supplementation on blood levels of IGF-system components (summarized in **Table 7.5**) (20;22-33). Besides the study described in this paper, only two other randomized, crossover trials evaluated the effects of isolated, red clover-derived isoflavones (20;28). Both studies observed no overall effect of isoflavones on circulating IGF-system components, which is in line with our results in postmenopausal women. In eight randomized, controlled studies intervention with soy foods or isolated soy protein containing isoflavones was compared to either control foods, isolated milk protein, or soy protein without isoflavones. In all of these studies protein intake markedly increased, both in the intervention group and the control group. Consequently, six out of eight studies observed increased blood levels of IGF-I in both groups (22-25;29;33), which is likely due to IGF-I increasing effects of essential amino acids in animal and soy protein (34;35). In only one study the increase in IGF-I levels between the two intervention groups differed significantly (23). Based on this qualitative review it can be concluded that isoflavones do not substantially increase or decrease blood levels of IGF-I.

Effects of isoflavone supplementation on normal colorectal tissue mRNA expression of IGF-system components have not been previously studied in humans. In our trial, no differences in mRNA levels of IGF-I, IGF-II, IGF-IR and IGFBP-3 were observed between the isoflavone intervention and the placebo treatment. In contrast, in *in vitro* studies of

Table 7.5. Human intervention studies on the effect of soy protein / isoflavones on blood levels of IGF-system components

Author, year	Study population[a]	Sample size	Study design[b]	Isoflavone intervention[c]	Duration (mo)	Protein intake	Results
Wangen, 2000 (22)	Healthy women (pre + post)	31	Cross	ISP LI (65) vs HI (130) vs C (8)	3	Increase in all 3 periods	Pre: Increase in IGF-I in LI (+11%, ns), not HI (vs C) Post: No change in HI and LI vs C. Increase in IGF-I in C (+18%) and LI (24%), not HI (vs baseline). No marked change in IGFBP-3
Khalil, 2002 (23)	Healthy men	46	Par	IP SP (88) vs MP	3	Increase in both groups	Increase in IGF-I after both SP (+65%) and MP (+14%, ns) Increase significantly greater after SP than MP
Adams, 2003 (24)	Men and women (post); high risk CRC	150	Par	ISP HI (83) vs C (3)	12	-	No effect on IGF-I and IGFBP-3 Increase in IGF-I (+8%) in both groups combined vs baseline
Arjmandi, 2003 (25)	Healthy women (post)	42	Par	IP SP (88) vs MP	3	Increase in both groups	Increase in IGF-I after SP (+68%) and MP (+36%) No significant difference between the two groups
Hussain, 2003 (26)	Male PC patients	39	Pilot	SIS (200)	3 to 6	-	No effect on IGF-I and IGFBP-3
Spentzos, 2003 (27)	Male PC patients	17	Pilot	ISP HI (114)	5	-	Increase in IGF-I (+22%) No effect on IGFBP-3
Campbell, 2004 (28)	Healthy women (pre + post)	23	Cross	CIS (86) vs P	1	No difference between periods	No effect on IGF-I, IGFBP-1, and IGFBP-3
Arjmandi, 2005 (29)	Healthy women (post)	62	Par	SF (60) vs CF	12	Increase in both groups (higher in CF)	Increase in IGF-I after SF (+26%) and CF (+13%) Increase in IGFBP-3 after SF (+5%), not CF No significant difference between the two groups
Gann, 2005 (30)	Healthy women (pre)	154	Par	ISP (+Ca) HI (88) vs C	3	Increase in both groups	No effect on IGF-I and IGFBP-1 Decrease in IGFBP-3 (-2%) in both groups combined vs baseline
Maskarinec, 2005 (31)	Healthy women (pre)	196	Par	SF (50) vs RD	24	Increase in both groups (higher in SF)	No effect on IGF-I, IGFBP-3, and molar ratio Positive association between urinary isoflavone excretion and serum IGF-I

(Table 7.5 continued)

Author, year	Study population[a]	Sample size	Study design[b]	Isoflavone intervention[c]	Duration (mo)	Protein intake	Results
Woodside, 2006 (32)	Healthy women (pre)	10	Pilot	SF (80[d])	0.25	-	Increase in IGF-I (+71%), IGFBP-3 (+18%), and molar ratio (+50%)
Vrieling, 2007 (20)	Men; high risk CRC	37	Cross	CIS (84) vs P	2	No difference between periods	No effect on total IGF-I, free IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 Inverse association between equol and IGF-I
Dewell , 2007 (33)	Male cancer patients	93	Par	LP+ISP (133) vs C (24)	12	Increase in LP+ISP vs C	Increase in both groups in IGF-I (LP+ISP: +18%; C: +11%) and IGFBP-3 (LP+ISP: +6%; C: +7%). No significant difference between the two groups Increase in IGFBP-1 in LP+ISP (+33%), not in C (-4%) No effect on IGFBP-2
Vrieling, 2007 (this paper)	Women (post); high risk CRC	34	Cross	CIS (84) vs P	2	No difference between periods	No effect on total IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3

[a] Pre = premenopausal, post = postmenopausal, CRC = colorectal cancer, PC = prostate cancer; [b] Cross = randomized, crossover study, Par = randomized, parallel study, Pilot = uncontrolled pilot study; [c] ISP = Isolated Soy Protein, HI = High Isoflavones, LI = Low Isoflavones, C = Control, IP = Isolated Protein, SP = Soy Protein, MP = Milk Protein, SIS = Soy-derived Isoflavone Supplement, CIS = Clover-derived Isoflavone Supplement, P = Placebo, SF = Soy Foods, CF = Control Foods, RD = Regular Diet, LP = Lifestyle Program, including very-low fat vegan diet, exercise. Isoflavone content in mg/day (between brackets); [d] Total phytoestrogen content, including isoflavones.

androgen-responsive prostate cancer cells genistein, daidzein and equol were all found to inhibit IGF-IR mRNA expression consistently even at relatively low levels of exposure comparable to human dietary intake (36;37). In human colon cancer cells genistein, but not daidzein, was found to inhibit IGF-IR protein levels and IGF-IR signalling at relatively high pharmacological concentrations (38). We could not confirm this finding *in vivo* in colorectal tissue. However, a limitation to our tissue study is that only one colonoscopy was performed in each participant. Therefore, tissue effects of isoflavones could only be evaluated between individuals and not within individuals. Substantial variability in tissue mRNA expression levels in colorectal biopsies of the same individuals sampled at different colorectal locations has been observed, with markedly higher expression of IGF-system components in the rectum as compared to the proximal colon (A. Vrieling, unpublished data). To evaluate colorectal tissue mRNA expression of IGF-system components in future studies, multiple colonoscopies with multiple biopsies at fixed locations in each participant are necessary, preferably in a cross-over design.
Adams et al. evaluated soy isoflavone effects on colorectal epithelial cell proliferation in a parallel trial (*n* = 91, men and women) comparing soy-protein powder (83 mg isoflavones/d) with ethanol-extracted soy-protein powder (containing 3 mg isoflavones/d). A colonoscopy was performed before and after the 12-month intervention period. No reduction in proliferation was observed. Surprisingly, an increase in cell proliferation measures in the sigmoid colon was found, which was opposite to the predicted direction (39). As mentioned previously, isoflavones in the context of a soy protein food may have different or even opposite effects as the effects hypothesized for isolated isoflavones. We were not able to study proliferation markers in our tissue specimens as the biopsies had not been oriented optimally to allow cutting slides containing full length crypts, which is needed for determination of proliferation (labelling index).

In conclusion, isolated isoflavones did not influence serum concentrations and tissue mRNA expression of IGF-system components in our randomized, placebo-controlled, double-blinded intervention study in postmenopausal women at increased risk of colorectal cancer. These results suggest that the increased serum IGF-I concentrations observed in previous studies investigating soy food or soy protein supplementation are most likely due to soy protein itself, and not to isoflavones. Potential effects of isoflavones on IGF-IR signaling in target tissues such as breast, colorectum, and prostate need further study.

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Part 2
MOLECULAR STUDIES

Insulin-like growth factor (IGF)-system mRNA quantities in normal and tumor breast tissue of women with sporadic and familial breast cancer risk

8

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Abstract

The Insulin-like Growth Factor (IGF)-system plays a role in breast cancer susceptibility as well as in growth and progression of breast carcinomas. So far, findings have been based on serum IGF-I levels and semi-quantitative assessment of IGF-system expression levels in model systems and human tissue. Quantitative data on mRNA expression in different types of human breast tissue are lacking.

Breast tissue samples (n = 83) were available from 72 women. Messenger RNA expression of IGF-I, IGF-II, and their receptors (IGF-1R and IGF-2R) was assessed by real-time RT-PCR. We found a large variation in mRNA levels. Expression of each gene was significantly higher in normal tissue than in tumor tissue (median for normal and tumor tissue, respectively (arbitrary units); IGF-I: 25.2 and 1.4; IGF-II: 5.9 and 0.6; IGF-1R: 0.18 and 0.07; IGF-2R: 1.8 and 0.9; $p < 0.0001$, Mann-Whitney test). Interestingly, in tumor tissue from patients with a strong family history of breast cancer, expression of both receptors was higher than in sporadic patients (IGF-1R: 0.13 and 0.05, $p = 0.04$; IGF-2R: 1.1 and 0.8, $p = 0.04$). For cancer-free controls, expression of IGF-II and IGF-2R in normal breast tissue was also higher in women with a family history of breast cancer than in women without such a family history (IGF-II: 7.2 and 1.5, $p = 0.02$; IGF-2R: 2.6 and 1.5, $p = 0.09$).

Our study quantitatively shows that mRNA expression levels of IGF-system components in the breast are generally higher in normal tissue compared with tumor tissue, and higher in tissue from women with a family history of breast cancer. A basis has therefore been created for studies aimed at understanding IGF as a breast cancer risk factor, the relationship between IGF-systems in serum and tissues, and effects of lifestyle factors on the IGF-system.

Introduction

Insulin-like growth factor (IGF)-I and IGF-II are growth factors essential for normal growth and development (1). Both IGF-I and IGF-II are known to act in an endocrine, paracrine and autocrine fashion. Model system and *in vitro* studies have shown that components of the IGF-system play a role in malignant transition of breast epithelial cells as well as in breast tumor growth (2). Binding of IGF-I or IGF-II to the IGF type 1 receptor (IGF-1R) activates intrinsic tyrosine kinase activity and triggers several signal transduction pathways, resulting in increased proliferation and reduced apoptosis (3). Conversely, binding of IGF-II to the IGF type 2 receptor (IGF-2R) causes degradation of IGF-II, and the IGF-2R is therefore thought to act as a tumor suppressor gene (4). Availability of IGF-I and IGF-II to bind to the IGF-receptors is largely dependent on IGF binding proteins (IGFBPs) (5).

High serum IGF-I levels are associated with increased risk of breast cancer, especially in premenopausal women (6-9). Additionally, women at high risk of breast cancer due to a family history of breast cancer have higher serum IGF-I levels than women without a family history of breast cancer (10). Circulating IGF-I is mainly synthesized by the liver, following stimulation by growth hormone (11). However, many other tissues also produce small amounts of IGF-I, and animal studies have suggested that tissue IGF-I may also contribute to circulating IGF-I levels (12).

Protein and mRNA expression of components of the IGF-system in human breast tissue have typically been studied using qualitative or semi-quantitative methods. Results from these studies are inconsistent and inconclusive with respect to mRNA expression levels in normal and malignant breast tissue. To investigate the plausible causative link between susceptibility (i.e., high serum IGF-I levels and cancer susceptibility) and tumor induction and promotion (i.e., tissue expression and subsequent cancer risk), quantitative data on the mRNA expression levels of IGF-system components in breast tissue are essential. The objective of our study was to quantify the levels of mRNA expression of IGF-I and -II, and both IGF-receptors in the breast, and to assess whether they differ in both normal and tumor breast tissue of women with and without a family history of breast cancer.

Materials and methods

Tumor and normal breast specimens

Breast tissue from a total of 72 anonymized women was used: 25 sporadic breast cancer patients, 27 breast cancer patients with a strong family history of breast cancer, 8 cancer-free controls without a known family history of breast cancer who underwent breast reduction surgery, and 12 cancer-free controls with a strong family history of breast cancer who underwent prophylactic mastectomy (**Table 8.1**). From 8 women more than one tissue sample was available, resulting in a total number of 83 tissue samples available for this study (35 normal tissue samples and 48 tumor samples). All breast tissue specimens were obtained during surgery/prophylactic surgery at the Netherlands Cancer

Table 8.1. Descriptive characteristics of the study population (*n* = 72) and available tissue samples (*n* = 83)

	Breast cancer patients			Cancer-free women	
	Sporadic	Strong family history		Breast reduction	Strong family history
		Tumor	Normal		
Patient characteristics					
Number of individuals (*n*=72)	25	27		8	12
Age at surgery (mean ± SD)	44 ± 8	41 ± 8		-[c]	42 ± 10
BRCA1/2 status (% carrier)	-[c]	67		-[c]	83
Tissue characteristics					
Number of tissue samples (*n*=83[a])	25	23	14	8	13
Normal tissue/normal breast (*n*=26)			5	8	13
Normal tissue/tumor breast (*n*=9)			9		
Tumor tissue (*n*=48)	25	23			
Tumor grade (%, grade III)	84	78			
Cell type (median, range)[b]					
Tumor cells	65, 40-90	60, 30-90	0	0	0
Normal epithelial cells	0, 0-20	5, 0-60	90, 60-90	90, 0- 90	90, 50-90
Stromal (i.e. all other) cells	25, 10-60	25, 10-50	10, 10-40	10, 10-100	10, 10-50

[a] from 8 individuals more than 1 sample was available for study (7x 2 samples and 1x 5 samples), see Figures 2 and 3; [b] percentage cell type in breast tumor samples of breast cancer patients, and normal breast tissue samples of cancer-free women, respectively; [c] not available

Institute/Antoni van Leeuwenhoek hospital, with the exception of the normal breast tissue samples from breast reduction surgery which were obtained at the University Medical Center of Maastricht. All tissue samples were snap-frozen in liquid nitrogen and kept at -80°C until use. Histopathological review of all tissue specimens was performed on H&E-stained snap-frozen tissue sections. The percentage of normal epithelial cells, tumor cells, and stromal cells (i.e., all cells of non-epithelial origin) was scored for all tissue samples.

RNA isolation and RT reaction

Total RNA was extracted from 83 tissue samples (30x 30µm slides) using RNAzolB (Campro Scientific). Total RNA (100 ng) was reverse-transcribed to generate first strand cDNA (total volume 60 µl) using random hexamers and Superscript II. The RT reaction was performed at 42°C for 90 minutes, followed by heating at 95°C for 2 minutes.

Real-time PCR

Real-time PCR was used to quantify the levels of mRNA expression of IGF-I, IGF-II, IGF-1R, and IGF-2R in tissue samples. Primers and probes for these reactions were designed using Primer Express software (Applied Biosystems, PE) (**Table 8.2**). Primers were chosen in two adjacent exons, and the fluorescent labeled probes were selected to partially encompass both exons, to avoid DNA contamination and amplification of the homologous insulin and

Table 8.2. Primers and probes for real-time PCR

Gene	Exon	Primer / probe[a]	Sequence
IGF-I	1,2	F	5'-AGCAGTCTTCCAACCCAATTATTTA-3'
		R	5'-AGATGCGAGGAGGACATGGT-3'
		Probe	5'-TCTTCACCTTCAAGAAATCACAAAAGCAGCA-3'
IGF-1R	8,9	F	5'-AAGGCTGTGACCCTCACCAT-3'
		R	5'-CGATGCTGAAAGAACGTCCAA-3'
		Probe	5'-TTCGCACCAATGCTTCAGTTCCTTCC-3'
IGF-II	8,9	F	5'-CCGTGCTTCCGGACAACTT-3'
		R	5'-GGACTGCTTCCAGGTGTCATATT-3'
		Probe	5'-CCCAGATACCCCGTGGGCAAGTTCT-3'
IGF-2R	34,35	F	5'-GCAGACATGCACTCTCTTCTTCTC-3'
		R	5'-GAGACAAGTCAACAATAGAGCTTCCA-3'
		Probe	5'-CCTGCGAGCAAGCGACCGAATG-3'

insulin receptor genes.

PCRs were carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions (40 cycles). The content of IGF-I, IGF-II, IGF-1R, and IGF-2R transcripts has been normalized to the content of the 'housekeeping gene' β-actin. A second 'housekeeping gene' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed in 50 of 83 tissue samples to evaluate the validity of normalization to β-actin content. As the quantity of both genes was highly correlated (Spearman rank correlation coefficient: $r = 0.91$, $p < 0.0001$) all results are normalized to β-actin (GAPDH data not shown). For β-actin and GAPDH PCRs primer and probe mixtures from Applied Biosystems were used under the same conditions as described above.

Standard curves were generated using serially diluted solutions of cDNA from MCF-7 cells for IGF-1R, IGF-2R, β-actin, and GAPDH. For IGF-I and IGF-II standard curves were generated using cDNA from a breast tumor sample known to contain high levels of IGF-I and IGF-II. All PCR assays were conducted in duplicate for each sample, and mean values were used in subsequent analyses. For a subset of samples ($n = 21$) repeated measurement of the β-actin quantity was undertaken, for which we observed a high correlation between assays (Spearman rank correlation coefficient: $r = 0.98$, $p < 0.0001$).

Data analysis

Normalization of mRNA expression levels of IGF-I, IGF-II, IGF-1R, and IGF-2R to the expression level of β-actin results in a dimensionless value (arbitrary units). Relative

mRNA expression of all four genes was not normally distributed in our samples, and transformation of the data did not result in a sufficiently normalized distribution. Therefore, relative mRNA expression levels in our samples are described by the median expression and the range (minimum and maximum value). All statistical analyses were conducted for three selections of the samples: (1) including all 83 samples, (2) excluding those samples for which duplicate experiments of a specific gene resulted in large variation, that is, coefficient of variation >25%, mostly due to very low expression (5, 7, 3, and 9 samples excluded for IGF-I, IGF-II, IGF-1R, and IGF-2R respectively), (3) excluding those samples for which the expression of β-actin was more than 20-fold lower than the mean β-actin expression in all samples (21 samples that were considered to have marginal mRNA content were excluded). The results did not markedly differ for the three sets of samples. All results are reported for the second set (2) of samples only. For four individuals more than one sample of the same tissue type was available; the mean expression in those samples was used in the statistical analyses.

Differences in relative mRNA expression of IGF-I, IGF-II, IGF-1R, and IGF-2R between two categories of samples (e.g., normal versus tumor) were evaluated using the Mann-Whitney test for non-parametric data, on SPSS (version 10.0) software. The Spearman rank correlation coefficient was calculated to estimate the correlation between mRNA expression of these four genes.

Results

Messenger RNA expression was assessed in 83 breast tissue samples of 72 women who had breast surgery and who were similar in age (**Table 8.1**). In women with a strong family history of breast cancer, 67% of the breast cancer patients and 83% of the cancer-free women were known BRCA1 or BRCA2 gene mutation carriers. The majority of tumors was grade III, and contained on average 65% (range 30-90%) tumor cells. Normal breast tissue samples generally contained 90% (range 50-90%) normal epithelial cells.

Figure 8.1 shows the relative IGF-I, IGF-II, IGF-1R, and IGF-2R mRNA expression in normal breast tissue and tumor breast tissue samples, separately for individuals with and without a positive family history. We observed a large variation in relative mRNA expression of both IGFs, with at least a 200-fold difference between the 5^{th} and 95^{th} percentile in mRNA expression of IGF-I and IGF-II, and only a 40-fold and 10-fold difference between the 5^{th} and 95^{th} percentile in IGF-1R and IGF-2R expression, respectively. Noteworthy, the within-individual variation in expression of IGF-system components in tissue samples of the same type was also of substantial magnitude, though much smaller than the overall between-individual variation (**Figure 8.2**).

The median relative mRNA expression of all four genes was significantly higher in normal tissue samples as compared to tumor tissue (Mann–Whitney test, $p < 0.0001$), although some tumor samples clearly overexpress IGF-1R (Figure 1). This difference between normal and tumor tissue was similar in the group of individuals with a strong family history

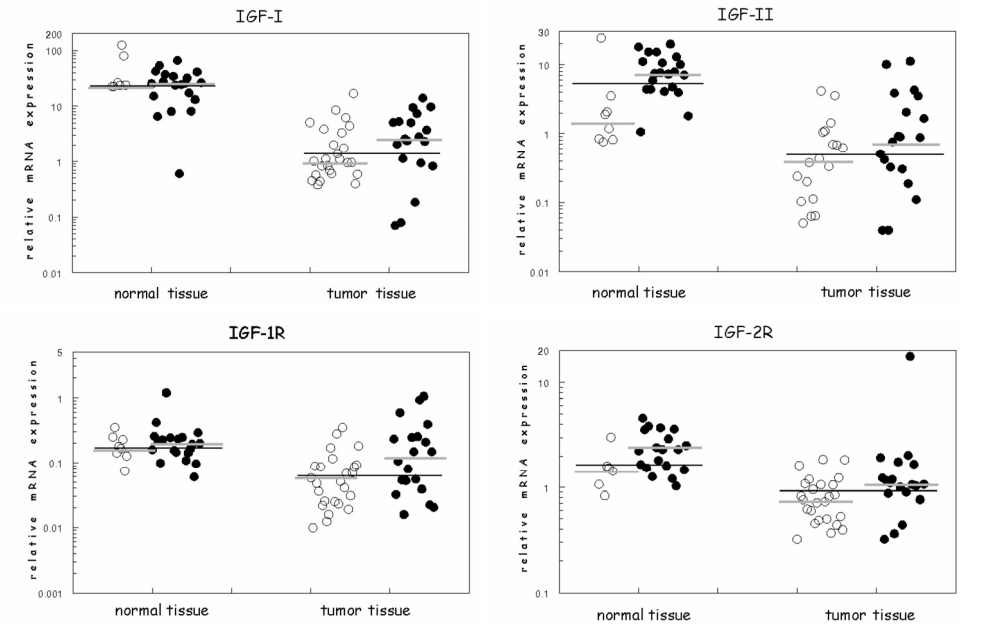

Figure 8.1. (a-d) Distribution of relative mRNA expression of IGF-system components in normal and tumor breast tissue, normalized to the content of β-actin in each sample (i.e., expressed in arbitrary units, not to be compared between genes). IGF-II, *n* = 6 no sample available. *Open circle* is individual with no known family history of breast cancer; *filled circle* is individual with a strong positive family history of breast cancer. Normal tissue of women with a positive family history includes: normal tissue adjacent to tumor, normal tissue from normal breast in cancer patient, and normal tissue from cancer-free women. Horizontal lines are the median expression levels in the groups.

of breast cancer and the group without such a family history. Interestingly, tumor tissue from patients with a strong family history of breast cancer expressed higher relative levels of IGF-1R and IGF-2R mRNA than tumor tissue from sporadic patients (median; IGF-1R: 0.13 and 0.05, p = 0.04; IGF-2R: 1.06 and 0.76, p = 0.04; Mann-Whitney test). Relative IGF-I and IGF-II expression in tumor tissue did not significantly differ between these two groups (p = 0.23 and p = 0.27, respectively). We also compared the expression of IGF-system components in normal breast tissue of women with and without a known family history of breast cancer. Relative IGF-II and IGF-2R mRNA expression was higher in normal tissue of women with a positive family history as compared to women who had undergone breast reduction surgery ('sporadic' controls) (median; IGF-II: 7.4 and 1.5, p = 0.005; IGF-2R: 2.30 and 1.48, p = 0.06). No significant difference in relative mRNA expression of IGF-I and IGF-1R genes was observed between these two groups (p = 0.61 and p = 0.82, respectively). Relative mRNA expression of IGF-system components in normal breast tissue from tumor-containing breasts was similar to normal breast tissue of 'cancer-free' women (data not shown).

Possibly, differences in expression between tissue samples can be explained by the predominant cell type in a tissue sample. Therefore, we tested whether expression in

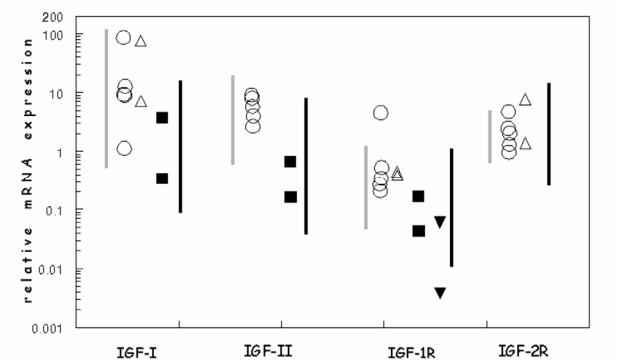

Figure 8.2. Within-individual variability in relative mRNA expression of IGF-system components in breast tissue samples of the same type in four individuals (arbitrary units, not to be compared between genes). Each individual is depicted by one type of marker; *closed marker* is tumor tissue, *open circle* is normal tissue adjacent to tumor, *open triangle* is normal tissue from cancer-free individual. Vertical lines represent the range of expression in all normal breast tissue samples (gray lines) and all breast tumor samples (black lines). The mean expression in the samples of each of these four individuals is included in the data-analyses comparing groups (Figure 1), including the range depicted by the vertical lines.

tumor samples differed depending on the proportion of tumor cells or normal epithelial cells in a sample. Although no significant differences in expression between categories based on percentage of a specific cell type were observed, median expression of IGF-system genes was somewhat higher in tumor samples with a high percentage of normal epithelial cells (i.e., ≥25%) as compared to tumor samples with a low percentage of normal epithelial cells (i.e., <10%), especially for IGF-I for which we observed a five-fold difference in median expression (data not shown).

For four individuals both tumor and normal breast tissue samples were available, which allowed us to confirm the results of the unmatched series in a small series of matched samples (**Figure 8.3**). Indeed, within each individual the relative mRNA expression level of IGF-I and IGF-II in the tumor tissue was markedly lower than the expression in the normal tissue sample. Interestingly, IGF-1R expression was found to be higher in the tumor tissue than in the normal tissue in two individuals. Though numbers are small, these detailed data give an impression of the consistency of differences between tissue types described so far, with the differences found within persons.

Finally, we studied whether these components of the IGF-system are co-regulated, in which case a high correlation between mRNA expression of certain components would be expected. **Table 8.3** shows the correlation between the relative mRNA expression of IGF-I, IGF-II, IGF-1R, and IGF-2R in normal and tumor breast tissue. No strong correlation between expression of these genes in normal breast tissue samples was observed. The strongest correlation was found between IGF-I and IGF-II mRNA expression in breast tumor samples (Spearman rank correlation coefficient: $r = 0.78$, $p < 0.0001$). In tumor samples

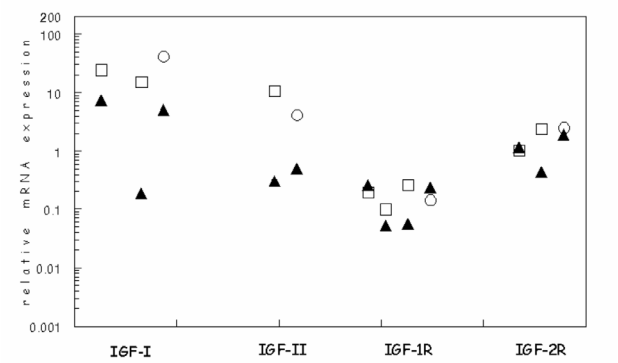

Figure 8.3. Within-individual comparison of relative mRNA expression of IGF-system components between normal tissue and tumor tissue samples (arbitrary units, not to be compared between genes). Samples of one individual are depicted in a vertical order. Different markers reflect tissue types: *closed triangles* are tumor tissue, *open squares* are normal tissue from the contra-lateral breast without tumor, *open circles* are normal tissue adjacent to tumor.

the expression of IGF-1R was also significantly positively correlated with the expression of both IGF-I and IGF-II (r = 0.47 and r = 0.55, respectively, $p < 0.001$). Interestingly, this positive association between mRNA expression of IGF-1R and its primary ligands IGF-I and IGF-II was restricted to tumor tissue from patients with a strong family history of breast cancer (r = 0.75 and r = 0.75, $p < 0.0001$, respectively).

Table 8.3. Correlation matrix (Spearman's correlation coefficients) of the association between mRNA expression of IGF-I, IGF-II, IGF-IR, and IGF-IIR in 83 breast tissue samples

	IGF-I	IGF-II	IGF-1R	IGF-2R
Normal breast tissue samples (n = 35)				
IGF-I	1.00			
IGF-II	0.04	1.00		
IGF-1R	-0.10	0.35	1.00	
IGF-2R	0.32	0.22	0.16	1.00
Breast tumor tissue samples (n = 48)				
IGF-I	1.00			
IGF-II	0.78[a]	1.00		
IGF-1R	0.47[a]	0.55[a]	1.00	
IGF-2R	0.14	0.12	0.19	1.00

[a] $p < 0.001$; In tumors from sporadic patients: r = 0.68 (IGF-I and IGF-II), r = 0.05, (IGF-1R and IGF-I), and 0.34 (IGF-1R and IGF-II), and in tumors from patients with family history of breast cancer: r = 0.80 (IGF-I and IGF-II), r = 0.75, (IGF-1R and IGF-I), and r = 0.75 (IGF-1R and IGF-II).

Discussion

Our data provide the first quantitative evaluation of IGF-system mRNA levels, using Real

Time RT-PCR, in normal and tumor breast tissue from women with and without a family history of breast cancer. Overall, substantial inter- and intra-individual variation in expression levels of these genes was found in breast tissue samples. Expression levels of IGF-I, IGF-II, IGF-1R, and IGF-2R genes in our samples were generally higher in normal tissue as compared to tumor tissue, also shown for tissue samples from the same individual. Moreover, relative mRNA expression of some components of the IGF-system was also higher in tissue from individuals with a strong family history of breast cancer than in tissue from individuals without such a history.

Studies to date have been inconsistent with regard to mRNA expression of IGF-system components in breast tumor tissue and normal breast tissue. The methods used in these studies, such as RT-PCR and RNAse protection assays, have been (semi-) quantitative, using imaging techniques to quantify mRNA levels of one or more IGF-system components in small numbers of samples. With respect to IGF-I, studies suggest that IGF-I mRNA is expressed in the majority of normal breast tissue samples, as well as in some tumors (13;14). IGF-II mRNA was found to be expressed in both tumor and normal tissue, although inconsistency remains with respect to the level of expression in both tissue types (13;15-17). IGF-1R mRNA expression has been found in the majority of breast tumors (13). Early studies using binding assays have shown much lower levels of IGF-1 receptor in normal breast tissue as compared to breast tumors (18;19). The IGF-2R, which is thought to act as a tumor-suppressor, is expressed in the majority of breast tumors (13). In our study, the majority of breast tumor samples expressed low quantities of IGF-I and IGF-II, as compared to normal breast tissue. Our quantitative data therefore confirm with higher precision what some previous studies (15;16) already suggested (14-16;20): that in normal breast tissue high mRNA levels of IGF-I and IGF-II are present, creating an environment which may stimulate cell proliferation and inhibit apoptosis. Furthermore, we observed high IGF-1R mRNA expression in normal breast tissue and large variation in IGF-1R expression in tumor breast tissue. Noticeably, about one-fourth of all breast tumor samples in our study expressed levels of IGF-1R above the median expression in normal tissue samples, suggesting increased potential for tumor growth promoting signal transduction. This inconsistency with the early results from binding assays, may be explained by non-specific binding (e.g., other receptors or binding proteins), or by differences between mRNA expression and protein expression. However, our results are in line with some immunohistochemical studies of IGF-1R protein levels (21;22). We also observed lower expression of IGF-2R mRNA in tumor samples as compared to normal samples, which would be in line with loss of tumor-suppressor function.

So far, no studies have been published on potential differences in tissue IGF-system components in relation to mammary carcinogenesis between women with and without a family history of breast cancer. We found higher expression of some IGF-system components in normal breast tissue and breast tumor tissue from individuals with a strong family history of breast cancer, as compared to these two breast tissues from individuals without such a family history. A cross-sectional epidemiological study of determinants of

serum IGF-I levels suggested that healthy women with a family history of breast cancer have higher serum levels of IGF-I than women without a family history of breast cancer (10). Possibly, the association between serum IGF-I levels and breast cancer risk indeed plays an important causative role in women with a family history of breast cancer. Our findings also suggest a significant role for the IGF-system in inherited breast cancer etiology, although it remains to be elucidated whether serum IGF-I and IGF-II concentrations and expression of IGF-system components in breast tissue are associated or co-regulated. Interestingly, the breast cancer susceptibility gene BRCA1 is known to suppress the IGF-1R promotor activity in several cell lines (23), which may explain our finding of higher levels of IGF-1R mRNA in breast tumor tissue of patients with strong family history of breast cancer (of which 67% are known BRCA1/2 mutation-carriers).

Our observations with respect to differences between normal and tumor tissue, and between women with and without a breast cancer family history, may in part be due to differences in the predominant cell types in these tissues. Others have used *in situ* hybridization techniques to localize mRNA expression, and found IGF-I mRNA expression to be limited to stromal cells (14;24). Inconsistency remains with respect to the type of cells in which IGF-II mRNA is expressed (24-26). Within our breast tumor samples those samples with a relatively high percentage of normal epithelial cells (i.e., relatively low percentage of tumor cells) expressed higher levels of IGF-I. Due to very limited variation in the percentage of stromal cells we were not able to study whether this was associated with expression of IGF-system components. As the proportion of different cell types was very similar in tissue of women with and without a family history, this could not explain the differences in expression found between these groups.

Exposure of human breast tissue to IGF-signaling is potentially determined by a combination of local mRNA expression, post-transcriptional modifications, protein stabilization and/or degradation of IGFs and its binding proteins and receptors, as well as levels of circulating IGF-system components. We observed higher mRNA expression of the IGF-system genes in normal breast tissue than in breast tumors. In contrast, higher protein levels of IGF-I and IGF-1R in tumor as compared to normal breast tissue were found in most but not all studies using immunohistochemistry, radioimmunoassays or binding assays (18;19;21;22;27;28). So far, no studies have extensively investigated the co-regulation of exposure to IGF-system components within relevant tissues, such as breast tissue, and in the circulation. We found IGF-1R mRNA expression in tumor tissue to be highly correlated to the mRNA expression of IGF-I and IGF-II. Our results, as well as those from others (20), therefore suggest that at least some components of the IGF-system are co-regulated. One study found increased serum concentrations of IGF-I, IGFBP-1, and IGFBP-3, accompanied by increases in pancreatic tissue IGF-I, IGFBP-1, IGFBP-3 and IGF-1R in patients with pancreatic cancer compared with controls (29). Unfortunately, no individually matched data were shown. Of much interest is the observation that mice lacking hepatic IGF-I synthesis (LID-mice, generated using the cre/loxP system), have 75% reduction in circulating IGF-I levels but normal expression in other tissues, apparently contributing to

the serum IGF-I levels (12). LID-mice receiving colon tumor transplants develop less colon tumors and liver metastases than control mice receiving colon tumor transplants (30). It remains to be seen what the effect of lowering circulating IGF-I levels would be on cancer risk in humans.

The IGF-system is a potential target for cancer prevention and it is therefore of major importance to unravel the determinants of tissue and circulating IGF-system components, their association with each other, their combined effect on cancer risk, and their susceptibility to alteration aimed at decreasing cancer risk in populations at high risk. To achieve this, analyses of large series of breast tissue samples should be conducted, providing quantitative data on mRNA expression and protein content of all components of the IGF-system. These should then be correlated to serum concentrations of IGFs and their binding proteins (IGFBPs), as well as to markers of proliferation and apoptosis, and clinical markers such as mammographic breast density. Additionally, more studies should be conducted on the potential determinants of both tissue and circulating IGF-system components, such as caloric intake, body mass index, physical activity, and consumption of dietary or synthetic chemopreventive substances such as phyto-estrogens/selective estrogen-receptor modulators, anti-oxidants (e.g. lycopene), vitamin D, and retinoic acid.

Our data provide a basis for future studies on the association between tissue IGF-systems and circulating IGF-system components, as well as for studies on the effects of lifestyle factors on these components.

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Expression of Insulin-like Growth Factor system components in colorectal tissue and its relation with serum IGF levels

9

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Submitted

Abstract

Objective: The Insulin-like Growth Factor (IGF)-system has been implicated in colorectal carcinogenesis. The objective of this study was to measure expression levels of various IGF-system components in different locations of the colorectum, and to investigate whether these tissue IGF expression levels are correlated with circulating serum IGF-I and IGF-II concentrations.

Design, setting and patients: Biopsies from macroscopically normal mucosa at four locations in the colorectum (ascending, transverse, and sigmoid colon, rectum) and a fasting serum sample were obtained from 48 asymptomatic patients at increased risk of colorectal cancer.

Main outcome measures: Expression levels of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 messenger RNA (mRNA) in tissue were quantitatively evaluated using real-time RT-PCR. Expression of IGF-IR protein in the ascending colon and rectum tissue specimens was assessed semi-quantitatively by immunohistochemistry. Serum IGF-I and IGF-II concentrations were determined using immunometric assays.

Results: With the exception of IGF-IIR, mRNA levels of all the IGF-system components investigated, as well as IGF-IR protein expression, were significantly higher in the rectum compared with the ascending colon ($p \leq 0.001$). Circulating IGF-I and IGF-II concentrations did not correlate with any of the parameters studied in colorectal tissues.

Conclusions: Our results indicate that in humans IGF-system components are differentially expressed in the colorectum. Moreover, our findings suggest that local and circulating components of the IGF-system are differentially regulated. Our data underline the importance of taking into account the colorectal location when investigating dietary or pharmacological effects on colorectal tissue mRNA expression of IGF-system components.

Introduction

The insulin-like growth factor (IGF)-system has an important role in normal as well as tumor cell growth (1). IGF-system components are expressed in most tissues, including the colorectum (2). The liver is the principal source for IGF-I and IGF-II protein in the blood circulation, where the majority of IGFs exist in a ternary complex with IGF binding protein (IGFBP) -3 and the acid labile subunit (ALS). The remainder of the circulating IGFs is associated with other binding proteins, or present in a free form (≤ 1%) (3). Locally produced and circulating IGFs can be released from their binding proteins by IGFBP proteases (4). These free IGFs can bind to the cellular membrane IGF-I receptor (IGF-IR), resulting in stimulation of proliferation and inhibition of apoptosis through the phosphatidylinositol 3'-kinase (PI3K/Akt) and mitogen-activated protein kinase (MAPK) pathways (4). The binding of IGF-II to the IGF-IIR leads to degradation of the IGF-II protein (4).

Local IGF expression is thought to affect colorectal carcinogenesis via an autocrine or paracrine pathway. The IGF-IR and particularly IGF-II have often been found to be overexpressed in human colorectal tumor as compared to normal colorectal tissue (5-7). Furthermore, IGF-I protein (8) and messenger RNA (mRNA) (9), IGF-II protein (5), and IGF-IR mRNA (9) expression in colorectal tumors have been significantly positively associated with proliferation. High circulating IGF-I and IGF-II concentrations have also been associated with increased risk of colorectal cancer (10). Furthermore, *in vivo* studies revealed that in mice with liver deleted IGF-I expression (LID mice), circulating IGF-I concentrations were reduced by 75%, and colorectal tumor development, growth and metastases were decreased (11). Both lines of evidence suggest an important role of the IGF-system in the development of colorectal cancer.

At present, no data are available on mRNA expression levels of IGF-system components throughout the colon. Moreover, it is unknown whether circulating IGF-I and IGF-II proteins directly affect colorectal tumor growth in humans through IGF-IR binding and activation, whether they influence local tissue expression of IGF-system components (e.g. upregulation of IGF-I, IGF-II, or IGF-IR), or whether they are reflective of tissue IGF-system component expression and thereby act as a biomarker of tissue IGF-system component bioactivity. To investigate this in more depth, we quantitatively evaluated mRNA expression levels of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 in normal colorectal tissues obtained from four locations in the colorectum. Additionally, we studied their relationship with serum IGF-I and IGF-II concentrations.

Materials and Methods

Study population

Men (40 to 75 years of age) and postmenopausal women (50 to 75 years of age) with a personal history of colorectal adenomas or at least one first degree family member with a history of colorectal cancer were selected from medical registries and pathology databases of four hospitals in the Netherlands (the Antoni van Leeuwenhoek hospital in Amsterdam, the Gelderse Vallei hospital in Ede, the Slotervaart hospital in Amsterdam, and the Sint Antonius hospital in Nieuwegein). Exclusion criteria were a history of cancer, familial adenomatous polyposis syndrome, familial Li Fraumeni syndrome, chronic inflammatory bowel disease, diabetes mellitus, acromegaly, significant liver or renal disease, (partial) bowel resection, non-remissive celiac disease, diverticulitis, other severe comorbidity, and laxative abuse. Forty-eight asymptomatic patients (32 males and 16 females) who were scheduled to undergo a surveillance colonoscopy between November 2003 and October 2005 participated in our study. The study protocol was approved by the Medical-Ethical Committees of all participating centers.

Colorectal tissue specimens

Colonoscopy was performed after whole-gut lavage with 4 L of a macrogol: Klean-Prep (Norgine BV, Amsterdam, the Netherlands) or Coloforte (Ipsen Farmaceutica BV, Hoofddorp, the Netherlands). Colorectal biopsies were obtained with a standard-sized flexible endoscopic forceps and collected from four locations: the ascending colon, transverse colon, sigmoid colon, and rectum. At each location, four biopsies from macroscopically normal mucosa were taken. Two biopsies were snap-frozen in liquid nitrogen and stored at -70^{o}C until preparation of RNA. The other two biopsies were formalin-fixed and paraffin-embedded for immunohistochemical analyses.

RNA isolation and RT reaction

Total RNA was extracted from the tissue samples using RNAzolB (Campro Scientific). Total RNA (5 μg) was reverse-transcribed (RT) to generate first strand complementary DNA (cDNA) (total volume 100 μl) using random hexamers and Superscript II. After 10 min RT for extension of the hexameric primers, the RT reaction was performed at 42°C for 60 min, followed by heating at 98°C for 5 min. cDNA was diluted 1:1 with RNAse free water before real-time RT-PCR.

Real-time RT-PCR

Real-time RT-PCR was used to quantify the levels of mRNA expression of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 in all tissue samples obtained from the ascending colon (n = 2*48, 2 biopsies from 48 individuals) and rectum (n = 2*48), and in a selection of tissue samples obtained from the transverse (n = 2*28) and sigmoid colon (n = 2*28). Primers and probes for these reactions were designed using Primer Express software (Applied Biosystems, PE) (**Table 9.1**). Primers were chosen in two adjacent exons, and the fluorescent-labeled

Table 9.1. Primers and probes for real-time PCR

Gene	Exon	GenBank accession no.	Primer / probe[a]	Sequence
IGF-I	1,2	NM_000618	F	5'-AGCAGTCTTCCAACCCAATTATTTA-3'
			R	5'-AGATGCGAGGAGGACATGGT-3'
			Probe	5'-TCTTCACCTTCAAGAAATCACAAAAGCAGCA-3'
IGF-IR	8,9	X04434	F	5'-AAGGCTGTGACCCTCACCAT-3'
			R	5'-CGATGCTGAAAGAACGTCCAA-3'
			Probe	5'-TTCGCACCAATGCTTCAGTTCCTTCC-3'
IGF-II	8,9	NM_000612	F	5'-CCGTGCTTCCGGACAACTT-3'
			R	5'-GGACTGCTTCCAGGTGTCATATT-3'
			Probe	5'-CCCAGATACCCCGTGGGCAAGTTCT-3'
IGF-IIR	34,35	XM_004237	F	5'-GCAGACATGCACTCTCTTCTTCTC-3'
			R	5'-GAGACAAGTCAACAATAGAGCTTCCA-3'
			Probe	5'-CCTGCGAGCAAGCGACCGAATG-3'
IGFBP-3	2,3	BC018962	F	5'-AGAGCACAGATACCCAGAACTTCTC-3'
			R	5'-ATTGAGGAACTTCAGGTGATTCAGT-3'
			Probe	5'-CATTTCTCTACGGCAGGGACCATATTCTGTCT-3'

[a] F = forward primer, R = reverse primer.

probes were selected to partially encompass both exons, to avoid DNA contamination and amplification of the homologous insulin and insulin receptor genes. PCRs were carried out using the ABI Prism 7700 Sequence Detection System (Applied Biosystems) according to manufacturer's instructions (50 cycles). The content of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 transcripts was normalized to the content of the 'housekeeping gene' ß-actin. A second 'housekeeping gene' glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was assessed to evaluate the validity of normalization to ß-actin content. For ß-actin and GAPDH, PCR primer and probe mixtures from Applied Biosystems were used under the same conditions as described above. As the quantity of both genes was highly correlated (Spearman rank correlation coefficient: $r = 0.78$, $p < 0.001$), all results were normalized to ß-actin (GAPDH data not shown). Standard curves were generated using serially diluted solutions of cDNA from a mixture of one normal breast sample and 11 cell lines. All PCR assays were conducted in duplicate for each sample and the values were averaged.

Immunohistochemistry

Immunohistochemistry (IHC) for IGF-IR was performed on normal tissue biopsies obtained at the colon ascendens ($n = 2*46$) and the rectum ($n = 2*43$). For some individuals no paraffin material was available ($n = 2$ and $n = 5$, respectively). Slides of 3 mm from formalin-fixed paraffin-embedded tissue samples were pretreated (antigen retrieval) with citrate buffer (0.01 M citric acid, in distilled water (pH 6.0); 15 min at 100 ^{0}C). They were then incubated with a primary mouse anti-human monoclonol antibody against the α-

subunit of the IGF-IR (1:100, Lab Vision Corporation / Neomarkers, USA; overnight at 4 ^{0}C). Antibody binding was visualized using the two-step Power Vision$^+$ detection system (ImmunoVision Technologies, USA). Replacement of the primary antibody with normal antibody diluent (Scytek Laboratories, USA) served as negative control. Sections of human placenta were used as a positive control. Evaluation of immunohistochemical staining was performed in a blinded and independent manner by two investigators (AV and AB). Cases of disagreement were reviewed jointly to reach a consensus score. Since the IGF-IR is universally expressed in colorectal tissue, IHC results of IGF-IR were scored semi-quantitatively for staining intensity (0 = absent/very weak, 1 = weak, 2 = moderate, 3 = strong). Hereby, no distinction was made between staining of cell membrane or cytoplasm.

Determination of IGF-I and IGF-II in serum

Fasting blood samples drawn approximately one hour before colonoscopy were frozen and stored at -30^{o}C until further analysis. Serum total IGF-I was measured using an immunometric technique on the Advantage Chemiluminescense System (Nichols Institute Diagnostics, San Juan Capistrano, USA). Serum IGF-II concentrations were determined in Sep-Pak C18 extracts of serum by a radioimmunoassay (RIA), as described previously (12).

Statistical methods

The mean mRNA expression level of two biopsies taken at the same location was used in statistical analyses. Normalization of mRNA expression levels of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 to the expression level of ß-actin resulted in a dimensionless value (arbitrary units (AU)). Relative mRNA expression of all five genes was not normally distributed in our samples, and transformation of the data did not result in a sufficiently normalized distribution. Therefore, non-parametric tests were used.

A Friedman test for non-parametric data was used to determine whether relative mRNA expression of these five genes differed among the four colorectal locations. To evaluate which locations were significantly different from each other a Wilcoxon signed ranks test was performed. The same test was used to determine differences in IGF-IR protein expression between the ascending colon and the rectum.

Spearman rank correlation coefficients were used to assess the correlation between tissue mRNA expression of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3, and between tissue mRNA expression and serum IGF-I and -II concentrations. A Pearson correlation coefficient was calculated to estimate the correlation between serum IGF-I and IGF-II concentrations. Whether differences in IGF-IR protein expression in tissue specimens (i.e., score 1, 2, and 3) related to differences in corresponding serum concentrations of IGF-I or –II was estimated with a Kruskal –Wallis test.

All statistical analyses were conducted by three different approaches: 1) including all samples, 2) excluding those samples for which duplicate experiments of a specific gene in one biopsy resulted in large variation, i.e. a coefficient of variation >20% being mostly

related to very low expression, 3) excluding those samples for which the expression level of ß-actin was more than 20-fold lower than the mean level for all samples. Since the results for these different approaches did not appear to be markedly different, only data that included all samples are presented. All statistical analyses were performed using SPSS 12.0 (SPSS Inc., Chicago, IL, USA).

Results

Study population

This study included 48 asymptomatic individuals (32 males, 16 females) who underwent a colonoscopy and from whom colorectal biopsies and a fasting serum sample were obtained. Sixteen individuals had a family history of colorectal cancer and a personal history of colorectal adenomas, 11 individuals had a family history of colorectal cancer only, and 21 individuals had a personal history of colorectal adenomas only. Mean (± SD) age was 58 ± 9 years.

Concordance of IGF mRNA expression in normal colorectal tissue

IGF-I, IGF-II, IGF-IR, IGF-IIR and IGFBP-3 mRNA expression was detected in all samples at all locations. To assess the intra-individual variation in mRNA expression of IGF-system components in two colorectal biopsy specimens sampled at the same location, intraclass correlation coefficients (ICC) as a measure of concordance were calculated. The ICC approaches the value 1 when there is hardly variation in mRNA expression within individuals compared to the variation between individuals. Concordance was highest for IGFBP-3 (range 0.66 to 0.76, except in the sigmoid colon; 0.17), lower for IGF-I, IGF-IR, and IGF-IIR (range 0.29 to 0.68) again with the exception of the sigmoid colon (range 0.02 to 0.25), and poor for IGF-II mRNA expression (range 0.03 to 0.13, except in the transverse colon; 0.57) (**Table 9.2**). Concordance was highest for the rectum (range 0.46 to 0.76). To obtain a more accurate measure of mRNA expression levels for the various IGF-system components, the mean value of two biopsy specimens was used in further analyses.

Table 9.2. Intraclass correlation coefficients for mRNA expression of IGF-system components for two biopsies obtained at the same location in the colorectum

Location	Ascending	Transverse	Sigmoid	Rectum
mRNA	colon	colon	colon	
IGF-I	0.38[a]	0.29	0.13	0.46[b]
IGF-IR	0.47[b]	0.46[a]	0.25	0.68[b]
IGF-II	0.03	0.57[b]	0.05	0.13
IGF-IIR	0.46[b]	0.36[c]	0.02	0.67[b]
IGFBP-3	0.75[b]	0.66[b]	0.17	0.76[b]

[a] $p \leq 0.01$, [b] $p \leq 0.001$, [c] $p \leq 0.05$.

IGF mRNA expression in normal colorectal tissue

Median mRNA expression was significantly higher in the rectum as compared to the ascending colon for all IGF-system components investigated (median for rectum and ascending colon, respectively (arbitrary units); IGF-I: 0.12 and 0.07; IGF-II: 0.15 and 0.06; IGF-IR: 1.20 and 0.70; IGFBP-3; 0.58 and 0.17; $p \leq 0.001$), except for IGF-IIR mRNA that was equally expressed at both locations (1.96 and 1.86) (**Figure 9.1**).

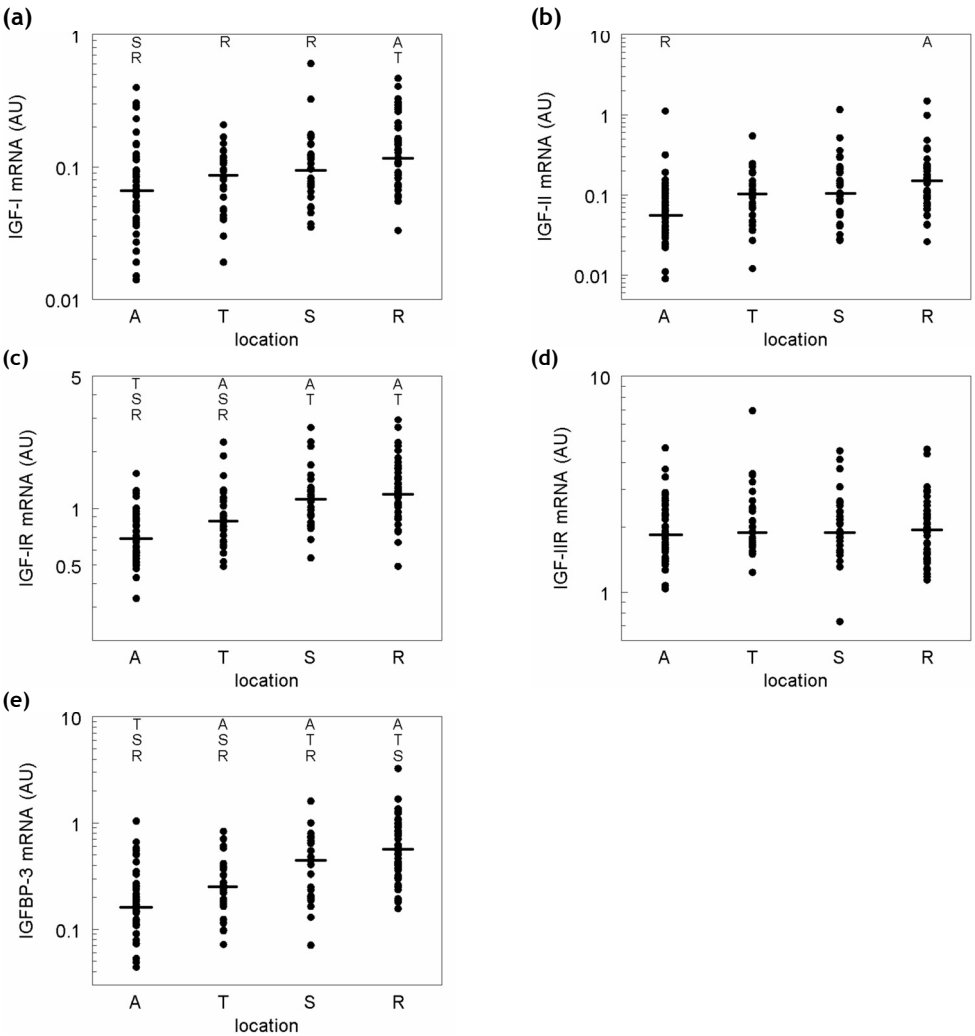

Figure 9.1. IGF-I (a), IGF-II (b), IGF-IR (c), IGF-IIR (d) and IGFBP-3 (e) mRNA expression relative to the housekeeping gene beta-actin (arbitrary units, AU) at four locations in the colorectum; the ascending colon (A), the transverse colon (T), the sigmoid colon (S), and the rectum (R).The location symbols above the different locations indicate a significant difference in mRNA expression between that location and the other locations ($p \leq 0.005$).

Levels of IGF-I, IGF-II, IGF-IR and IGFBP-3 mRNA in the transverse and sigmoid colon were intermediate to those in the ascending colon and rectum. In the total set of biopsies, obtained from all locations, all IGF-system components were significantly correlated ($p \leq 0.001$) (**Table 9.3**).

Table 9.3. Correlation matrix (Spearman's correlation coefficient) of the association between mRNA expression of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 in 304 colorectal tissue biopsy samples[a]

	IGF-I	IGF-II	IGF-IR	IGF-IIR	IGFBP-3
IGF-I	1.00				
IGF-II	0.43	1.00			
IGF-IR	0.64	0.53	1.00		
IGF-IIR	0.50	0.30	0.63	1.00	
IGFBP-3	0.63	0.67	0.62	0.39	1.00

[a] $p \leq 0.001$ for all IGF parameters.

IGF-IR protein expression in normal colorectal tissue

For 46 individuals IGF-IR protein expression could be evaluated in biopsies obtained from the ascending colon, whereas for 43 individuals rectal biopsies were available for analysis. IGF-IR protein staining was observed in the colonic crypts of all biopsies, both in the cytoplasm and in the cell membrane (**Figure 9.2**). In contrast, stromal tissue did not show any staining. Staining intensity was significantly higher in the rectum compared to the ascending colon (Figure 9.2; strong staining in 54% of the rectum and 24% of the ascending colon biopsies, respectively, $p \leq 0.001$), confirming the data on mRNA expression.

Relation between tissue IGF mRNAs and IGF-IR protein expression

For both the ascending colon and the rectum, there was no apparent relationship between the individual level of IGF-IR mRNA expression and the degree of IGF-IR protein expression **(Figure 9.3)**. Similarly, IGF-I, IGF-II, IGF-IIR, and IGFBP-3 mRNA expression levels did not correspond to the degree of IGF-IR protein staining.

Serum IGF-I and IGF-II concentrations

Mean (± SD) serum concentrations of IGF-I and IGF-II were 133 ± 50 μg/L and 480 ± 108 μg/L, respectively. These concentrations were within the normal range. Inter-individual variation in serum IGF-I and –II appeared to be rather large, i.e. five-fold and three-fold differences were found between the extreme concentrations of serum IGF-I and -II, respectively (serum IGF-I: range 54 – 277 μg/L and serum IGF-II: range 278 – 779 μg/L). Serum IGF-I concentrations correlated weakly but significantly with those of serum IGF-II (Pearson $r = 0.39$, $p = 0.007$).

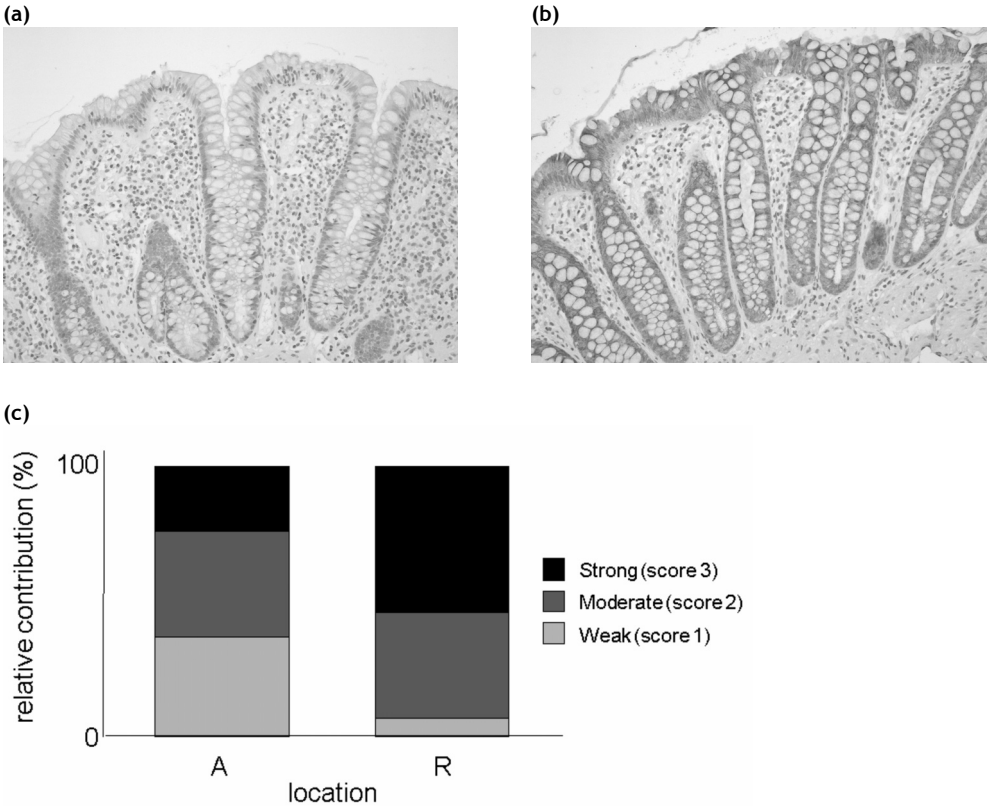

Figure 9.2. Immunohistochemical staining of the IGF-I receptor in representative sections of the ascending colon (a) (weak staining, score 1) and rectum (b) (strong staining, score 3). Relative distribution of the scores for intensity of positive cytoplasm and membrane staining combined in the ascending colon (A) and rectum (R), separately (c).

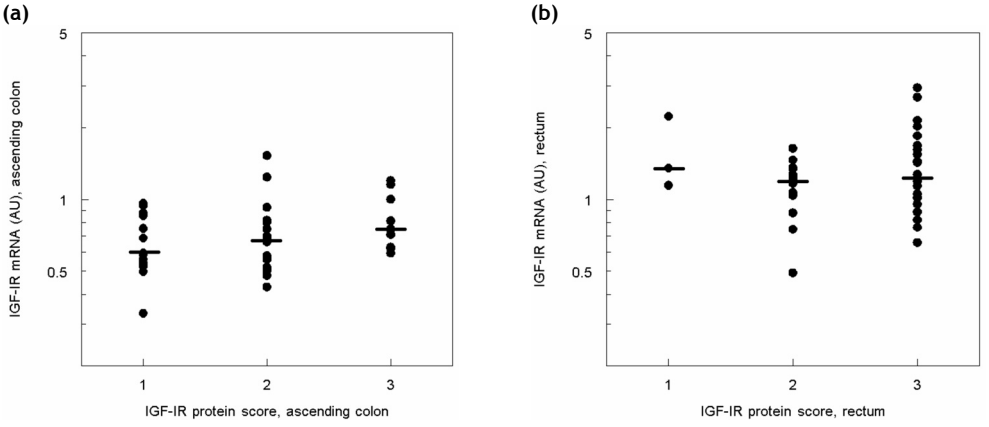

Figure 9.3. The relation between IGF-IR mRNA and protein expression in the ascending colon (a) and rectum (b).

Relation between serum IGF-I and IGF-II and tissue IGF mRNAs

To obtain a valid measure of overall colorectal tissue mRNA IGF expression, we first averaged the expression of 1) the ascending colon and rectum (n = 48; 4 biopsies per individual), and 2) all locations combined (n = 28; 8 biopsies per individual). For both series, serum IGF-I concentrations did not correlate with either tissue IGF-I, IGF-IR, IGF-II, IGF-IIR, or IGFBP-3 mRNA expression (range Spearman r = -0.21 to r = 0.14, data not shown). Similarly, no statistically significant correlations were observed for serum IGF-II with any of the IGF-system components either (range Spearman r = -0.15 to r = 0.17, data not shown). Since we observed that the various IGF-system components were differentially expressed in colorectal tissue, we also performed the correlation analysis for each tissue location separately. However, for each of the four locations neither serum IGF-I nor serum IGF-II concentrations were significantly associated with tissue mRNA expression of any of the IGF-system components, as illustrated in **Figure 9.4** only for serum IGF-I concentrations and tissue IGF-IR mRNA expression in the ascending colon and rectum, respectively.

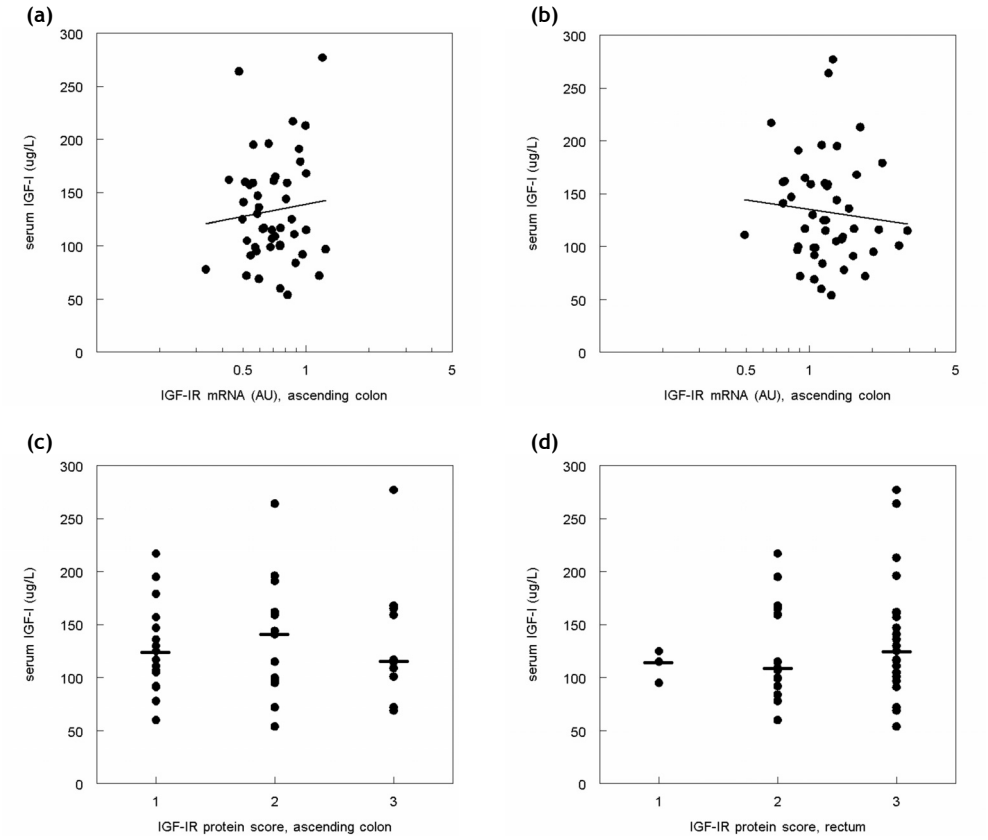

Figure 9.4. The correlation of serum total IGF-I concentrations with IGF-IR mRNA (a, b) and protein (c, d) expression in the ascending colon (a, c) and rectum (b, d).

Relation between serum IGF-I and IGF-II and IGF-IR protein expression
Serum IGF-I concentrations were not significantly different for individuals with biopsies with a weak, moderate, or strong IGF-IR protein staining, both for the ascending colon and the rectum, respectively (Figure 9.4). Serum IGF-II concentrations did not differ for the different IGF-IR protein staining categories either (data not shown).

Discussion

To our knowledge, this is the first study that demonstrates differences in mRNA expression of IGF-system components in human colorectal tissue derived from different anatomical sites. We could detect mRNA expression of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 in all colorectal tissue samples, indicating that our quantitative real-time RT-PCR technique was highly sensitive. Only few previous studies have used a similar quantitative technique to measure mRNA expression of IGF-system components in normal colorectal tissue, which was derived from normal tissue adjacent to tumor tissue in colorectal cancer patients (6;7;9;13). In these studies, a much higher between-tissue variation in mRNA expression of IGF-I, IGF-II, IGF-IR and IGFBP-3 was found compared to our findings in normal colorectal tissues from individuals without cancer. This may be due to tumor effects on the adjacent normal tissue (7). We also detected IGF-IR protein expression in all colorectal biopsies investigated. IGF-IR protein expression was not correlated with IGF-IR mRNA expression. This has been previously observed in breast tumor and normal breast tissue samples, and can be explained by the fact that many other intracellular signaling pathways may modify the final protein product (14).

The concordance of mRNA measurement in two biopsy samples obtained from the same location in the colorectum differed for the different IGF-system components. The concordance was highest for IGFBP-3, which has been previously shown to be equally expressed in normal epithelial and stromal cells of normal colorectal tissues adjacent to tumor tissue (13). The concordance was lower for the other IGF-system components, which may be due to their differential expression in different cell types. To our knowledge, no information with respect to cell-type specific mRNA expression of these IGF-system components in colorectal tissue is available.

The differential expression of IGF-system components observed in our study may be explained by specific functions of different parts of the colon and rectum (15). Differential colonic and rectal mRNA expression has also been observed for other genes in other studies (16;17). Our findings underline the importance of taking into account the colorectal location when investigating dietary or pharmacological effects on colorectal tissue mRNA expression of IGF-system components.
Circulating IGF-I and -II concentrations in our study population at increased risk for colorectal cancer were within the normal range with large interindividual variation, and

comparable with those reported in previous studies (10). However, even within this large range, no correlation was found with colorectal mRNA expression of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3. Moreover, no association with tissue IGF-IR protein expression was found. These results suggest that in humans circulating (endocrine) and local (autocrine / paracrine) compartments of IGF-system component expression levels are differentially regulated. However, we have to take in account that the relatively low concordance between biopsies obtained at the same location may have contributed to the fact that no correlations were observed with circulating IGF-I and -II concentrations.

The relation between circulating and local IGF-system components has primarily been investigated in animal models, which show variable results. In mice with liver deleted IGF-I expression (LID mice), circulating total IGF-I concentrations were reduced by 75%, whereas no changes in normal growth and IGF-I mRNA expression in various examined tissues were observed (i.e., heart, muscle, fat, spleen, and kidney) (18). These results indicate that circulating IGF-I concentrations and tissue IGF-I mRNA expression are not co-regulated. However, expression levels of IGF-IR mRNA and IGF-I mRNA levels in the colon were not studied. In contrast, energy and / or protein restriction and concomitant decreases in circulating IGF-I concentrations in rats reduced normal growth, and resulted in different effects on IGF-I and IGF-IR mRNA expression for different tissue types (19-24). Only two studies have investigated these effects in colorectal tissue (25;26), and showed a non-significant decrease in IGF-I mRNA and a significant increase in IGF-IR mRNA expression after chronic protein restriction (25). Changes in circulating IGF-I concentrations as well as in tissue IGF-I and IGF-IR mRNA expression were reversed by protein repletion (26). In the latter study, circulating IGF-I concentrations were significantly inversely associated with IGF-IR mRNA expression, suggesting a compensatory response to preserve IGF-IR signaling in colonic mucosa during protein restriction. Thereby, the results of these studies indicate that circulating IGF-I concentrations directly affect tissue mRNA expression levels of IGF-I and IGF-IR. However, fasting effects are different for rats as compared to humans, and therefore these results are difficult to compare (27).

In human studies, circulating IGF-I concentrations in patients with acromegaly were positively correlated with proliferation (28) and negatively correlated with apoptosis (29) of the normal colonic mucosa. Furthermore, circulating IGF-II concentrations in colorectal cancer patients were correlated positively with the proliferation of normal colonic mucosa adjacent to a colorectal tumor, but not with the proliferation of the tumor itself (30). IGF expression in these tissues was not determined. Unfortunately, we have not been able to investigate proliferation in our study, since biopsies were not orientated and of inadequate size to get sufficient scorable crypts. However, our results suggest that these previously observed increases in proliferation and decreases in apoptosis are direct effects of circulating IGF-I and -II by binding to and activation of the IGF-IR, and not due to

changes in expression levels of colorectal tissue IGF-system components. To definitively conclude whether circulating IGF-I and -II concentrations are directly associated with activation of the IGF-IR, determination of IGF-IR phosphorylation status would be necessary with antibodies directed against the phosphorylated IGF-IR or intermediates in the signal transduction pathway.

In conclusion, our results indicate that IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 are differentially expressed in human colorectal tissue. Neither circulating IGF-I nor IGF-II concentrations were associated with colorectal tissue mRNA expression of IGF-system components. Therefore, the relatively increased serum IGF-I and IGF-II concentrations observed in individuals who are at high risk to develop colorectal cancer are not likely to affect or to be reflective of normal colorectal tissue mRNA expression levels of IGF-system components.

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GENERAL DISCUSSION

10

The main aim of the studies described in this thesis was to investigate the effects of lycopene and isoflavone supplementation on circulating IGF-system components in premenopausal women at increased breast cancer risk, and in men and women at increased colorectal cancer risk. Additionally, we studied whether differences exist in expression levels of IGF-system components between normal and tumor tissues, and tried to elucidate the relation between circulating and tissue levels of IGF-system components. In this chapter, the main findings are summarized, methodological issues of our randomized placebo-controlled studies are discussed, and our findings are integrated, also considering the results of other studies. Finally, general conclusions are drawn, possible public health implications are mentioned, and recommendations for future research are given.

Main findings

Increased circulating IGF-I concentrations are associated with increased risk of premenopausal breast cancer and colorectal cancer, and may be influenced by dietary factors, e.g. lycopene and isoflavone intakes (review, **Chapter 2**). However, the low habitual dietary intake of lycopene and isoflavones in the Dutch female population investigated in our cross-sectional study (median intake ~3 mg/day and 0.15 mg/day, respectively) was not associated with circulating total IGF-I and IGFBP-1, IGFBP-2, and IGFBP-3 concentrations (**Chapter 3**).

To accurately investigate whether higher intake of lycopene and isoflavones affects circulating IGF-system components, we conducted randomized placebo-controlled trials with tomato-derived lycopene (30 mg/day) and red clover-derived isoflavones (84 mg/day) supplementation for two months (**Chapters 4-7**). The results of these trials are summarized in **Table 10.1.**

Table 10.1. The mean within-person cross-over differences (%) in circulating IGF-system components between the intervention (lycopene or isoflavones) and the placebo treatment for all studied groups, separately

	Increased breast cancer risk		Increased colorectal cancer risk			
	Lycopene		Lycopene		Isoflavones	
IGF-system components	Survivors (n=24)	Family history (n=36)	Men (n=40)	Women (n=31)	Men (n=37)	Women (n=34)
Total IGF-I	7.0	-3.3	-2.2	-3.2	-1.3	-2.0
Free IGF-I	6.3	**-7.6**	-[b]	-	-1.2	-
IGF-II	-	-	-1.2	5.4	4.0	-1.7
IGFBP-1[a]	-7.3	-2.4	-9.5	**21.7**	-7.1	-6.2
IGFBP-2	-5.1	2.4	**8.2**	7.8	3.4	11.0
IGFBP-3	**3.3**	-1.3	-0.9	1.7	1.0	-1.8

[a] medians; [b] - were not assessed; significant findings ($p \leq 0.05$) are displayed in bold.

Lycopene supplementation did not decrease circulating IGF-I concentrations in women at increased breast cancer risk and men and women at increased colorectal cancer risk (**Chapters 4-5**). Lycopene supplementation resulted in differential effects on circulating IGF-system components in breast cancer survivors and healthy women with a family history of breast cancer (**Chapter 4**). In the breast cancer survivors, circulating total IGF-I and IGFBP-3 concentrations were increased following lycopene supplementation (mean relative difference 7.0%, 95%CI -0.2% to 14.3%; and 3.3%, 0.7% to 6.0%, respectively). In contrast, in healthy women with a family history of breast cancer free IGF-I concentrations as a measure of bioavailable IGF-I were decreased after lycopene supplementation (mean relative difference -7.6%, 95%CI -14.6% to -0.6%). No changes were observed in circulating concentrations of other IGF-system components. In women at increased colorectal cancer risk, lycopene supplementation increased circulating IGFBP-1 concentrations (median relative difference 21.7%, $p = 0.01$) (**Chapter 5**). In both men and women at increased colorectal cancer risk, circulating IGFBP-2 concentrations were increased after lycopene supplementation (mean relative difference 8.2%, 95%CI 0.7% to 15.6%; and 7.8%, 95%CI -5.0% to 20.6%, respectively). Circulating IGF-II and IGFBP-3 concentrations in this population were not altered.

The effect of isoflavone supplementation on circulating IGF-system components was investigated in men and women at increased colorectal cancer risk (**Chapters 6-7**). Isoflavone supplementation did not affect circulating IGF-I concentrations in both men and women (mean relative difference -1.3%, 95%CI -8.6 to 6.0%; and -2.0%, 95%CI -8.0 to 3.9%, respectively), or any of the other IGF-system components. About 30-50% of individuals are known to be able to convert daidzein, one of the main isoflavone metabolites, to the more potent estrogenic metabolite equol (1). Interestingly, higher serum concentrations of equol were associated with decreases in serum IGF-I concentrations after isoflavone supplementation in our study in men ($r = -0.49$, $p = 0.002$) (**Chapter 6**). We also investigated the effect of isoflavone supplementation on mRNA expression of IGF-system components in normal colorectal tissue biopsies of women, but observed no differences between women on isoflavones and women on placebo (**Chapter 7**).

To obtain more insight in the role of locally expressed IGF-system components in breast carcinogenesis, we quantitatively determined mRNA expression of IGF-I, IGF-II, and their receptors in normal breast and breast tumor tissue (**Chapter 8**). We observed a higher expression of all components in normal breast compared with breast tumor tissue. IGF-receptor expression in normal breast and breast tumor tissue was higher in women with a family history of breast cancer compared with women without a family history.

Finally, we tried to elucidate whether circulating and local IGF-system components are co-regulated or independently regulated (**Chapter 9**). mRNA expression of IGF-system components was significantly higher in the rectum than in the ascending colon. Circulating IGF-I and IGF-II concentrations were neither correlated with normal colorectal tissue IGF-IR mRNA and protein expression, nor with IGF-I, IGF-II, IGF-IIR, or IGFBP-3 mRNA expression in the ascending colon and the rectum.

In conclusion, although circulating and local IGF-system components may both play an important role in breast and colorectal carcinogenesis, we did not find an association between circulating and colorectal tissue levels of these components. Lycopene supplementation may increase circulating IGFBP-1 and IGFBP-2 concentrations, thereby increasing bioavailable IGF-I. Isoflavone supplementation did not affect circulating and colorectal tissue expression of IGF-system components, but may decrease circulating IGF-I concentrations in equol producers only.

Methodological considerations

Previous studies investigating the effects of lycopene and isoflavones on circulating IGF-system components were primarily conducted in experimental animal models or in humans using an observational study design. Randomized, placebo-controlled, double-blinded intervention studies will provide the most definitive answer to whether such effects exist. In this paragraph, we discuss the main features of the randomized studies described in this thesis that are relevant for the interpretation of our results, i.e., the study population, study design, lycopene and isoflavone intervention dose, duration and compliance, IGF endpoints, and statistical analysis.

Study population

Interventions directed at lowering circulating IGF-I concentrations should be aimed at populations at increased risk of cancers shown to be associated with increased circulating IGF-I concentrations (e.g. prostate, premenopausal breast, and colorectal cancer), since these populations are most likely to benefit from such interventions. As circulating IGF-I concentrations decrease with ageing, and have also been inversely associated with risk of age-related diseases such as cardiovascular disease, osteoporosis, and neurodegenerative disease (2), targeting the IGF-system in the general population is not relevant in this stage of research and may be associated with adverse effects. We chose to conduct our studies in premenopausal women at increased breast cancer risk who constitute an important part of the patient population of the Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital, and men and women at increased colorectal cancer risk. In the latter group, we had the opportunity to collect normal colorectal tissue samples in addition to blood samples.

In contrast to what we initially planned, we decided not to conduct an isoflavone intervention trial in women at increased breast cancer risk. Although two recent meta-analyses of epidemiological studies showed that soy intake may be associated with a small decrease in breast cancer risk (3;4), *in vitro* and *in vivo* studies found both tumor promoting and protective effects of (soy) isoflavones on breast cancer (5). In experimental studies in humans no consistent effects of isoflavones on indicators of cell proliferation in normal breast tissue were found (5). However, we could not exclude the possibility that isoflavones may increase proliferation in existing breast cancer. If we had pursued this

intervention, we should have informed the women about possible adverse effects and include markers of breast tissue proliferation. This would undoubtedly have dramatically decreased response rates for this intervention study.

Selection and recruitment

The study population at increased breast cancer risk consisted of premenopausal breast cancer survivors and premenopausal women with a family history of breast cancer, younger than 50 years of age. The study population at increased colorectal cancer risk consisted of men aged 40 to 75 years and postmenopausal women aged 50 to 75 years with a personal history of colorectal adenomas, a family history of colorectal cancer (including few individuals with Hereditary Non-Polyposis Colorectal Carcinoma (HNPCC)), or both. Further eligibility criteria have been described in the methods section of the relevant chapters (**Chapters 4-7**).

The recruitment of participants for our intervention studies was more laborious and time-consuming than we had expected, which should be taken into account in designing future studies in similar populations. Initially, we started off with large numbers of potentially eligible breast cancer survivors that were selected from the medical registry of the Netherlands Cancer Institute - Antoni van Leeuwenhoek hospital, and women with a family history of breast cancer that were selected from a database of high-risk women undergoing surveillance. However, actual numbers of women that could be invited for participation in our study turned out to be much lower. This was due to the exclusion of a substantial part of women (55% and 30% for the two populations, respectively) that did not fulfil the selection criteria (e.g. second breast tumor, prophylactic mastectomy, no first degree family member diagnosed with breast cancer at age ≤50 years, peri- or postmenopausal status, irregular menses). Also after invitation for our study, not all women fulfilled the selection criteria (e.g. peri- or postmenopausal status, irregular menses) or could be contacted (~35% and 20% for the two populations, respectively) (flow charts, **Chapter 4**).

We initially planned to recruit the individuals at increased colorectal cancer risk in two hospitals only. However, to achieve sufficient numbers of participants within our study time frame, we needed to extend our study with two other hospitals. These hospitals eventually contributed almost half of our study population (**Appendix 8**). We used different selection strategies, which are unlikely to have affected our results since intervention effects were compared within individuals. However, when designing future studies in similar study populations it is relevant to know which selection strategies were most successful in our study. The selection of individuals with a history of colorectal adenomas was best performed by an electronic search (search terms ‘colon’ and ‘adenoma’) in the PALGA databank (Pathology Anatomical National Automated Archive) (6). An advantage of this selection procedure was that we could immediately select for adenomatous polyps. Electronic searches in the medical registry database required confirmation of polyp histology by medical records. In only one hospital, we could

specifically select for individuals with a family history of colorectal cancer or HNPCC by using an available screening list of this population, which was not possible with the other selection strategies. We developed a database of eligible individuals, including their advised date for colonoscopy, which was either extracted from their medical records or obtained from their gastroenterologist. In one hospital, a comparable medical registry database was already present. Every month, we selected those individuals that were due to undergo a surveillance colonoscopy within the next two months. Additionally, we asked the gastroenterologists to inform eligible patients who visited their outpatient clinic about our study. This latter strategy proved to have limited efficiency and resulted in only two included patients.

Response

Response rates for our intervention studies ranged from 22% to 48%, with the lowest rates in women at increased colorectal cancer risk who were not scheduled to undergo a surveillance colonoscopy and in premenopausal breast cancer survivors. The highest response rates were achieved in men at increased colorectal cancer risk (flow charts, **Appendix 9**). Response rates for the total population at increased colorectal cancer risk were similar for all four hospitals (response, **Appendix 8**). Non-response was mostly due to practical reasons such as time and distance, or to the emotional burden of participating in such an intervention study and visiting the hospital, and was of no importance in the interpretation of our results.

Heterogeneity

Within the populations at increased breast or colorectal cancer risk there was heterogeneity in background cancer risk, which may have resulted in variability in baseline levels of circulating IGF-system components. Differences in baseline levels may result in differential intervention effects on circulating IGF-system components. Women with a family history of breast cancer were previously found to have higher circulating IGF-I concentrations than women without a family history (7), which was confirmed in our studies (**Chapter 4**). Subgroup analyses in breast cancer survivors and women with a family history of breast cancer did reveal differential effects on serum IGF-system components after lycopene supplementation, and therefore separate report was warranted. This possibility had been accounted for in our power calculations.

Individuals with colorectal adenomas were previously shown to have significantly higher circulating IGF-I concentrations compared with control subjects without adenomas (8). In our studies, no differences in serum IGF-I concentrations were observed at the end of the first intervention period for 24 individuals with an adenoma diagnosis at colonoscopy compared with 92 individuals without a diagnosis of adenomas at colonoscopy. To our knowledge, no data are available comparing circulating IGF-I concentrations in individuals with a family history of colorectal cancer and individuals without a family history of colorectal cancer. Our data suggest that age-adjusted serum IGF-I concentrations were

higher in individuals with a family history of colorectal cancer than in individuals without a family history of colorectal cancer (age-adjusted beta = 0.184, p = 0.04). No clear differences between these subgroups were observed with respect to effects on serum IGF-system components. However, numbers were too small to draw any definitive conclusions. Therefore, it is recommended to separately investigate these subgroups in future studies.

Study design

We chose to use a cross-over study design (study scheme, **Chapter 7**), since the inter-individual variation in circulating IGF-system component concentrations is known to be large. Compared with a parallel design, the inter-individual variation is eliminated and consequently a smaller sample size is needed. Power calculations were based on the primary endpoint serum total IGF-I. On the assumptions of an intra-individual coefficient of variation of 11% in serum total IGF-I concentrations, a sample size of 26 individuals was required to detect a 10% difference (α=0.05) with a power of 90% between the two treatment periods in our primary endpoint. Intra-individual coefficients of variation for the other circulating IGF-system components were expected to be similar, except for IGFBP-1 that is known to be subject to more variation. Intra-individual variation in mRNA expression of IGF-system components in breast and colorectal tissue was unknown. Each of the intervention studies described in this thesis consisted of at least 24 individuals who completed the study protocol.

Drop-out rates were relatively high in both populations at increased breast cancer risk (~20%), and low in individuals at increased colorectal cancer risk (~5% for men and ~10% for women, respectively). Drop-out may be a problem in the interpretation of the results, when it is related to the intervention studied. In our studies, drop-out was evenly distributed over the different intervention periods and due to reasons unrelated to the intervention (e.g. practical reasons, hormonal factors), and is therefore unlikely to have affected our results. We were not able to include these drop-outs in our analyses, since intra-individual differences were analyzed using endpoint measurements from both intervention periods.

Carry-over effects of the lycopene and isoflavone interventions were not expected to be a problem in our studies, since lycopene and isoflavone concentrations in individuals starting with the intervention period had returned to baseline values at the start of the placebo period.

All blood samples were drawn under fasting conditions. In women, circulating IGF-system components, in particular IGFBP-1 may vary during the menstrual cycle (9-12). Therefore, all blood withdrawals in the premenopausal women at increased breast cancer risk were planned on days 3 to 5 of their menstrual cycle, when estradiol and progesterone levels are relatively stable. In our lycopene intervention study in men and our isoflavone intervention study in men and women, a surveillance colonoscopy was performed at the end of the first intervention period. Consequently, the fasting duration at the end of the first intervention period was longer than at the end of the second intervention period.

This has complicated the interpretation of our results on changes in serum IGFBP-1 concentrations, as will be described in the section on IGF endpoints.

Lycopene and isoflavone intervention; dose, duration, and compliance

In our studies, lycopene and isoflavones were not administered in the context of food products, but delivered as supplements. In order to closely resemble dietary intake, the two agents were derived from naturally occurring sources.

Lycopene supplements consisted of a tomato extract that, in addition to 15 mg lycopene, also contained the other bioactive compounds present in tomatoes (i.e. 1.5 mg phytoene, 1.4 mg phytofluene, 0.4 mg beta-carotene, and 5 mg tocopherols). This makes the composition of these supplements very comparable to that of tomato products. The lycopene dose (30 mg/day) we used in our intervention studies is relatively high compared with habitual dietary lycopene intake in countries in which relatively large amounts of tomatoes and tomato products are consumed. However, it can be easily reached consuming about two servings per day of foods rich in lycopene. Furthermore, this dosage has been used in many previous intervention studies resulting in similar increases in lycopene levels, and is considered to be safe (Observed Safe Level; 75 mg/day) (13). The 2-month duration of our lycopene supplementation was considered sufficient for observing effects on the IGF-system, since other human lycopene trials already observed biological effects within a few weeks (14).

Isoflavones were administered as red clover supplements. The composition of red clover supplements (25 mg biochanin, 8 mg formononetin, 4 mg genistein, 5 mg daidzein) is different from that of soy, the main food source of isoflavones which mainly consists of the glucosides genistin and daidzin. However, blood profiles of genistein and daidzein after administration are comparable (15).The isoflavone dose (84 mg/day) administered in our studies is within the range of habitual dietary intake in Asian countries. Additionally, genistein blood levels after 2 months of isoflavone supplementation were comparable to populations consuming a soy rich diet (15), and in the range of isoflavone concentrations known to produce biological effects (50-800 ng/mL isoflavones) (16). No adverse effects of this dose were apparent for short-term use, but data on long-term use are lacking (17;18). Our isoflavone supplementation study is likely to have been of sufficient duration to expect changes in circulating IGF-system components. In human trials on oral estrogens and SERMs, a decrease in serum IGF-I concentrations was observed within 2 months (19;20).

Compliance in women at increased breast cancer risk was relatively low (~80%) for such a short-term intervention study, also compared to the relatively high compliance in individuals at increased colorectal cancer risk (~95%). The fact that these women were younger and more time-restricted (e.g. jobs, young family) may have made adherence to the study regimen more difficult. A low compliance may lead to underestimation of the intervention effect. However, serum lycopene and genistein were significantly increased in the majority of participants, and excluding participants who were non-compliant did

not markedly influence the results in any of our studies. For both lycopene and isoflavones, high between-individual variation in blood levels was observed, which was unrelated to compliance (**Appendix 10**). This has also been found in other studies (15;21), and is most likely due to differences in metabolism between individuals.
Participants were blinded with respect to the intervention, intra-individual differences in self-reported daily intake of food products that are rich in lycopene and isoflavones were negligible for the two intervention periods, and no compensatory behavior in food choice could explain any effects additional to the intervention studied.

IGF endpoints

Our main endpoints were circulating IGF-system component concentrations. Additionally, we investigated tissue mRNA expression of IGF-system components and tissue protein expression of the IGF-IR.

Circulating IGF components

Circulating total IGF-I concentrations were chosen as our primary endpoint, since these are significantly positively associated with premenopausal breast cancer risk and colorectal cancer risk in epidemiological prospective cohort studies (22;23). Circulating total IGF-I concentrations are significantly positively associated with circulating unbound, free IGF-I concentrations and are one of the most important predictors of free IGF-I concentrations (24). Circulating free IGF-I concentrations are considered to reflect bioactivity of IGF-I: the amount of IGF-I available to bind and to activate the IGF-IR. Additionally, they are assumed to be related to tumor development, although this has been unproven thus far (25).
Circulating free IGF-I concentrations can be measured using two different methods, i.e. ultrafiltration and immunoradiometric assays (IRMA) (24). We used the IRMA, which is a commercially available kit detecting both unbound IGF-I and IGF-I readily dissociable from various IGFBPs. The IRMA is less laborious and has a larger precision than ultrafiltration, but measurements are dependent on time and temperature. In contrast, ultrafiltration is performed during steady state conditions and measures solely unbound IGF-I (24). It remains unknown which method best reflects the IGF-I supply to the tissues (26). Circulating free IGF-I concentrations were measured in the populations at increased breast cancer risk and in men at increased colorectal cancer risk participating in the isoflavone supplementation study. Circulating IGF-II concentrations were measured in the populations at increased colorectal cancer risk, since epidemiological studies have shown positive associations between circulating IGF-II and colorectal cancer risk. Other IGF-system components that may be related to increased cancer risk, i.e. IGFBP-1, IGFBP-2, and IGFBP-3, were measured in all studies. All IGF assays have been previously shown to be valid and reproducible.
In our lycopene intervention study in men and our isoflavone intervention study in men and women at increased colorectal cancer risk, insulin concentrations were lower at the

end of the first compared with the end of the second intervention period, due to differences in fasting duration. This has resulted in insulin-induced differences in IGFBP-1 concentrations. Since insulin-induced differences in IGFBP-1 concentrations may markedly vary among individuals, it is difficult to draw definite conclusions with respect to the relative contribution of the intervention to the observed alterations in circulating IGFBP-1 in these intervention studies. The effect of fasting on circulating IGFBP-2 concentrations has not been studied in great detail and is inconsistent, although it is much smaller than for IGFBP-1. For the interpretation of our results on circulating total IGF-I, free IGF-I, IGF-II, and IGFBP-3 concentrations differences in fasting duration were not a problem, since these components were previously found not to be affected by fasting up to 72 hours (27;28).

Tissue IGF mRNA expression

Tissue mRNA expression of IGF-system components in our studies was measured using quantitative real-time RT-PCR. This method allows absolute quantification of (low abundance) mRNAs in small tissue biopsies. Thereby, it is more sensitive than other mRNA quantification techniques, such as Northern blot and ribonuclease protection assays, which have been mostly used in previous studies (29).

The breast and colorectal tissue samples investigated in our study were surgical resection specimens and biopsies obtained at colonoscopy, respectively, containing both epithelial cells and stromal cells. Previous studies have shown differential mRNA expression of IGF-system components for different cell types in breast tissue. IGF-I mRNA expression has been primarily found in normal breast stromal cells, but not in normal epithelial cells and tumor tissue (30). IGF-II mRNA was predominantly expressed in stromal cells adjacent to tumor epithelial cells, but also within tumor epithelial cells (30). In our study, we did not separately investigate mRNA expression of IGF-system components in epithelial cells and stromal cells. However, adjustment for the proportion of epithelial cells in our samples did not significantly change our results. Adjustment for the proportion of stromal cells was not possible, due to very limited variation. We observed high intra-individual variation in mRNA expression, particularly for IGF-I and IGF-II. However, intra-individual variation was generally smaller than inter-individual variation. Differences in mRNA expression of IGF-system components that are expected to be large can be reliably studied using breast tissue samples, such as differences in IGF expression between normal breast and breast tumor tissue. However, the high intra-individual variation in mRNA expression of IGF-system components in breast biopsies implies that these are no suitable endpoint in intervention trials in which smaller differences are expected, unless large numbers of individuals using multiple biopsies are being investigated.

Information with respect to cell-type specific mRNA expression of IGF-system components in normal colorectal tissue is very scarce. To our knowledge, it is unknown whether mRNA expression of IGF-I, IGF-II and their receptors in colorectal tissue differs for different cell types. In one study, IGFBP-3 mRNA levels were quantitatively determined in epithelial and

stromal cells of normal colorectal tissues adjacent to a colorectal tumor by laser-capture microdissection (31). No differences in IGFBP-3 mRNA expression between stromal and epithelial cells were observed. In our study, we investigated the concordance of mRNA measurement of IGF-system components in normal colorectal tissue by sampling duplicate biopsies at the same colorectal location for all participants. Indeed, concordance was high for IGFBP-3 mRNA expression. This may be explained by the equal IGFBP-3 mRNA expression in normal epithelial and stromal cells as described before (31). Concordance was lower for the other IGF-system components: it was good for the IGF-IR and IGF-IIR, but poor for IGF-I and IGF-II. This suggests that these IGF-system components may be differentially expressed in different cell types. Although we did not evaluate cell type composition, we cannot exclude that this has affected our possibility to find correlations with circulating IGF-system component concentrations (**Chapter 9**). Similar as for the breast samples, mRNA expression of IGF-system components in single colorectal biopsies is no suitable endpoint in intervention trials, due to large intra-individual variation.

Statistical analysis

The only variable factor in a randomized cross-over study is considered to be the intervention. Other factors cannot be controlled for in statistical analyses but were found to be constant in our studies (i.e. hormonal factors, energy and protein intake, physical activity, weight, and waist and hip circumference).

Different methods for the statistical analysis of cross-over studies have been used in previous studies. In our studies, we calculated the mean cross-over difference for each of the IGF-system components for both intervention sequence groups, separately, and pooled these two mean cross-over differences. We tested whether the pooled cross-over difference significantly deviated from the null value with a t-test, using the pooled standard error of the mean cross-over differences (32). In this way, we were able to adjust for period effects by using the unweighted mean of the two intervention group means. However, in case of equal numbers of participants in both intervention groups, this test is equivalent to a standard one-sample t-test evaluating the deviation from null of the mean cross-over difference. In our study in women at increased breast cancer risk, numbers in both intervention groups were comparable, and both methods led to similar results. Therefore, we chose to report the standard one-sample t-test, since this test is more widely known. In the other studies, we reported the pooled method. Because of the skewed distribution of IGFBP-1, we used a sign test to investigate whether the *median* cross-over differences significantly deviated from the null value.

Integration of findings

In this paragraph, the results of this thesis are discussed and put into perspective, considering findings from other studies. We discuss our results on the effects of lycopene and isoflavones on circulating IGF-system components, the expression of IGF-system

components in normal breast and breast tumor tissue, and the association between circulating IGF-system components and colorectal tissue expression of IGF-system components. Additionally, we elaborate on the relevance of targeting the circulating and local IGF-system in individuals at increased cancer risk and individuals with (a history of) cancer.

Lycopene and IGF

Based on our results and those of previous studies, lycopene does not seem to be a promising agent for lowering circulating total IGF-I concentrations. Lycopene supplementation (30 mg/day) did neither affect serum total IGF-I and IGFBP-3 concentrations in the women with a family history of breast cancer, nor in the population at increased colorectal cancer risk. In contrast with our hypothesis, both serum IGF-I and IGFBP-3 concentrations in breast cancer survivors were significantly increased. This may be explained by differences in background cancer risk, but may also be due to chance. To our knowledge, our studies have been the largest and best-controlled studies evaluating the effects of tomato-derived lycopene supplementation on circulating IGF-system components published thus far. Four other intervention studies have addressed this issue (33-36), and have been extensively described in **Chapters 4 and 5**. Only one of these studies showed a significant 25% decrease in serum IGF-I concentrations after lycopene intervention (30 mg/day) with a mean duration of 10 days (36), whereas no effects on serum IGF-I and IGFBP-3 concentrations were observed in the other studies (33-35).

Our results do suggest that lycopene may increase circulating IGFBP-1 and IGFBP-2 concentrations. In postmenopausal women at increased colorectal cancer risk, serum IGFBP-1 concentrations were significantly increased (21.7%) after lycopene compared with placebo intervention. Also a significant increase in serum SHBG concentrations (7.8%) was observed. However, lycopene supplementation in premenopausal women at increased breast cancer risk did not affect either serum IGFBP-1 or SHBG concentrations. This may point to differential lycopene effects depending on the endogenous hormonal environment. Lycopene supplementation increased serum IGFBP-2 concentrations in both women and men at increased colorectal cancer risk, but this increase was only statistically significant in men. Also *in vitro* and *in vivo* studies have shown that lycopene may increase IGFBPs, thereby possibly reducing bioactive IGF-I, and reducing IGF-I signaling through the IGF-IR. To our knowledge, we are the first to evaluate the effect of lycopene supplementation on serum IGFBP-1 and IGFBP-2 concentrations in humans. Although the IGFBP-1 and IGFBP-2 increasing effects of lycopene observed in our study are of interest, more knowledge is needed about the function of these binding proteins in normal growth and carcinogenesis before further exploring their role as a chemopreventive target. On the one hand, increased IGFBP-1 and IGFBP-2 concentrations result in increased binding of IGF-I and thereby reduced bioavailable IGF-I (37). This is in line with their possible association with decreased colorectal cancer risk (38;39). On the other hand, IGFBP-1 and IGFBP-2 increase IGF-I half-life and are able to transport IGF-I out

of the blood stream to the target tissues, where it can induce IGF-IR signaling (37). Additionally, IGFBP-1 may have IGF-I independent effects (37).

Isoflavones and IGF

The results of our and previous studies do not provide evidence for a role of isoflavones in lowering circulating IGF-I concentrations. Our results in men do suggest that isoflavones may lower circulating IGF-I in populations that are capable of converting the isoflavone daidzein in the more potent estrogenic compound equol, which has not been previously described. We hypothesize that equol, because of its estrogenic properties, may exert a similar lowering effect on circulating IGF-I concentrations as estrogen and selective estrogen modulators. Confirmation of our findings in studies with larger numbers of equol producers is warranted. Intervention studies to date evaluating the effects of isoflavones on the circulating IGF-system have primarily compared soy foods or soy protein isolates containing isoflavones with either control foods, milk protein isolates, or soy protein isolates not containing isoflavones (40-50). These studies have been extensively reviewed in **Chapter 7**. It is likely that the increase in serum IGF-I concentrations observed in the majority of these studies is mainly due to soy protein, which may mask a potential IGF-lowering effect of isoflavones in equol producers (51). One pilot cross-over study has investigated the effect of a one-month isolated (red clover-derived) isoflavone supplementation (86 mg/day) on circulating IGF-system component concentrations (52). In line with our results, this study did not show any effects on serum IGF-I, IGFBP-1 and IGFBP-3 in both pre- (n = 16) and postmenopausal (n = 7) women (52).

We did not observe any effects of isoflavone supplementation on mRNA expression of IGF-system components in the ascending colon and rectum. To our knowledge, we are the first investigating the effects of isoflavone supplementation on colorectal tissue mRNA expression of IGF-system components. However, due to the parallel design and the high intra-individual variation in IGF mRNA expression, power was probably insufficient to observe differences between women on isoflavones and women on placebo. One study has investigated the effect of soy protein with isoflavones on colorectal cell proliferation, and did not observe any effects, except for an increased proliferation in the sigmoid colon (53).

IGF in normal and tumor tissue

In our study on mRNA expression of IGF-system components in breast tissue, we observed a higher expression in normal breast tissue (including breast tissue adjacent to the tumor) compared with breast tumor tissue for IGF-I, IGF-II, and both receptors. For one individual, a matched pair of breast tumor tissue and normal breast tissue adjacent to the tumor was available. Within this pair, mRNA expression was also relatively higher in normal breast tissue for all IGF-system components, except for IGF-IR. Results from other studies are inconsistent. Some studies have confirmed our results for IGF-I (54;55) and IGF-IR (55), suggesting a paracrine effect of IGF-I on breast tumor growth. However, other

studies did not show differential mRNA expression of IGF-I (56;57) and IGF-IR (54;56) in matched pairs of breast tumor tissue and normal breast tissue adjacent to the tumor.
To our knowledge, we are the first showing increased mRNA expression of IGF-system components in both normal breast tissue (IGF-II, IGF-IIR) and breast tumor tissue (IGF-IR, IGF-IIR) of women with a family history of breast cancer compared with women with no known family history of breast cancer. This suggests a causative role for local IGF-system components in breast cancer susceptibility of women with a family history of breast cancer.

Circulating and local IGF

In our study, circulating IGF-I and –II concentrations were not associated with local colorectal tissue mRNA expression of IGF-system components. These results suggest that although both circulating (endocrine) and local tissue (paracrine/autocrine) IGF-system components have been implicated in normal and tumor growth, they are unlikely to be co-regulated. Additionally, circulating IGF-I and –II concentrations are not likely to be markers of overall tissue expression of IGF-system components, thus reflecting paracrine/autocrine IGF-effects. Alternatively, our results suggest that the association between circulating IGF-I concentrations and cancer risk may be explained by a direct causal effect: binding of circulating IGF-I to the local tissue IGF-IR, thereby inducing downstream signalling cascades resulting in increased proliferation and inhibition of apoptosis. This needs to be investigated in future studies, using suitable antibodies against the phosphorylated IGF-IR.

IGF as a target in cancer prevention

Lowering circulating IGF-I concentrations is relevant for individuals at increased cancer risk, and no toxic effects are expected when using dietary components within a physiological dose range. Positive effects are also expected in cancer survivors and cancer patients. However, the type of intervention may be different for these populations, as is also illustrated by the unfavorable effects of lycopene on circulating IGF-I and IGFBP-3 in the breast cancer survivors in our study.
Targeting the IGF-IR to reduce IGF-signaling is only warranted in cancer patients in whom overexpression of the IGF-IR in tumor tissue has been found. IGF-IR targeting may be associated with adverse effects since the IGF-IR is ubiquitously expressed in most tissues. Selectively targeting the IGF-IR in tumor tissues is difficult, and co-targeting the insulin receptor may be a problem. The search for suitable IGF-IR inhibitors is ongoing.

Conclusions

In our randomized controlled cross-over studies, lycopene did not decrease circulating IGF-I concentrations in individuals at increased risk of premenopausal breast cancer or colorectal cancer. Isoflavones did not decrease circulating IGF-I concentrations in

individuals at increased colorectal cancer risk either. These results are in line with results from previous studies. Interestingly, lycopene increased IGFBP-1 concentrations in women at increased colorectal cancer risk and IGFBP-2 concentrations in men and women at increased colorectal cancer risk, thereby possibly decreasing bioavailable IGF-I. Additionally, our results suggest that isoflavones may decrease circulating IGF-I concentrations in equol producers only, which constitute about 30-50% of the Western population. Both findings need further investigation in future randomized controlled trials.
Although both circulating and local tissue IGF-system components play an important role in breast and colorectal carcinogenesis, we did not find an association between circulating and colorectal tissue levels of these components.

Implications for public health

The results of our studies and other evidence published to date do not provide support for health claims for lycopene and isoflavones in lowering circulating IGF-I concentrations in individuals at increased risk of premenopausal breast cancer or colorectal cancer.

Recommendations for future studies

The IGFBP-1 and IGFBP-2 increasing effects of lycopene found in our studies are of interest from a cancer chemopreventive perspective. However, more knowledge derived from *in vitro* and *in vivo* studies and epidemiological studies is needed about the precise roles of IGFBP-1 and IGFBP-2 in carcinogenesis before further investigating lycopene effects on these binding proteins in future randomized controlled cross-over trials. We do recommend including measurements of circulating IGFBP-1 and IGFBP-2 in planned or ongoing lycopene supplementation trials, for example with respect to prostate cancer risk. These trials should be adequately controlled for fasting duration.
It would be of great interest to further investigate our hypothesis that isoflavones may lower circulating IGF-I concentrations when they are metabolized into the more potent estrogenic metabolite equol. This can be studied in randomized controlled cross-over trials in equol producers. However, the recent development of equol supplements may provide another opportunity to investigate whether equol is capable of lowering circulating IGF-I concentrations. These supplements may ultimately be implemented as a chemopreventive tool in both equol and non-equol producers.
Moreover, it would be relevant to elucidate whether any of these dietary effects would be different for distinctive polymorphisms in the IGF genes.
There is a complex interaction between the IGF-system and other hormone and growth factor pathways (e.g. estradiol, vascular endothelial growth factor). With advancement of proteomic analysis techniques, it would be of interest to determine effects of lycopene and isoflavones on circulating concentrations of all these different pathway components

simultaneously. Similarly, it would be interesting to investigate lycopene or isoflavone effects on tissue expression using micro-array analyses. However, first more knowledge is needed with respect to cell type specific expression of local IGF-system components (e.g. by *in situ* hybridization, laser-capture microdissection). It would also be of interest to investigate whether individuals with high serum IGF-I concentrations have differential tissue expression of IGF-related growth factors than individuals with low serum IGF-I concentrations. Whether circulating IGF-I is causally related to cancer risk needs further investigation.

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Appendices

Appendix 1. Summary of prospective cohort (nested case-control) studies (*coh*) and case-control (*cc*) studies (including at least 100 cancer cases) on circulating IGF-I and IGFBP-3 concentrations and premenopausal breast cancer risk

Reference	Study design	Country	Cases / controls	Menopausal status[a]	IGF-I Adjusted RR high vs. low (95% CI)	IGFBP-3 Adjusted RR high vs. low (95% CI)	P for trend IGF-I / IGFBP-3
Hankinson, 1998 (28)	coh	US	76 / 105	Pre	2.33 (1.06-5.16)	-	0.08
				Pre[b]	2.88 (1.21-6.85)	-	0.02
			60 / 78	Pre, <50y	4.58 (1.75-12.0)	-	0.02
				Pre, <50y[b]	7.28 (2.40-22.0)	-	0.01
Toniolo, 2000 (29)	coh	US	172 / 486	Pre	1.60 (0.91-2.81)	1.18 (0.66-2.08)	0.09 / 0.63
			96 / 280	Pre, <50y	2.30 (1.07-4.94)	2.17 (0.99-4.76)	0.03 / 0.14
				Pre, <50y[b]	1.90 (0.82-4.42)	-	-
Kaaks, 2002 (30)	coh	Sweden	116 / 330	<50y	0.63 (0.29-1.39)	1.39 (0.35-2.91)	0.51 / 0.88
Krajcik, 2002 (31)	coh	US	66 / 66	Pre	3.49 (0.65-18.7)	2.37 (0.85-6.55)	0.05 / 0.08
				Pre[b]	2.01 (0.33-12.4)	5.28 (1.13-24.7)	0.24 / 0.03
Muti, 2002 (32)	coh	Italy	69 / 265	Pre	3.12 (1.13-8.60)	2.31 (0.97-5.53)	0.01 / 0.02
Yu, 2002 (33)	cc	China	161 / 167	Pre	2.29 (1.20-4.37)	3.71 (1.67-8.26)	0.01 / 0.002
				Pre[b]	1.92 (0.88-4.20)	2.69 (1.12-6.47)	0.24 / 0.02
Allen, 2005 (42)	coh	UK	70 / 209	Pre	1.19 (0.58-2.46)	0.60 (0.29-1.24)	0.63 / 0.02
				Pre[b]	1.71 (0.74-3.95)	0.49 (0.21-1.12)	0.21 / 0.07
Rinaldi, 2005[d] (43)	coh	US	138 / 259	Pre, <51y	1.93 (1.00-3.72)	2.03 (1.09-3.76)	0.02 / 0.02
				Pre, <51y[b]	1.56 (0.68-3.59)	1.49 (0.69-3.23)	0.23 / 0.33
				Pre, <51y[c]	2.43 (1.21-4.90)	0.97 (0.44-2.11)	0.005 / 0.68
Schernhammer, 2005[e] (44)	coh	US	218 / 281	Pre	1.6 (1.0-2.5)	1.2 (0.8-1.9)	0.07 / 0.71
			155 / 193	Pre, ≤50y	2.5 (1.4-4.5)	1.4 (0.8-2.4)	0.01 / 0.35
Rinaldi, 2006 (45)	coh	Europe	272 / 534	≤50y	1.03 (0.60-1.77)	0.92 (0.50-1.70)	0.81 / 0.69
			144 / 282	≤50y, ≥2y diag	1.86 (1.01-3.42)	1.61 (0.60-4.29)	-
Schernhammer, 2006 (46)	coh	US	239 / 478	Pre	1.02 (0.71-1.48)	1.17 (0.82-1.66)	0.87 / 0.90
				Pre[b]	0.92 (0.61-1.41)	1.23 (0.83-1.82)	0.48 / 0.79
			129 / 275	Pre, ≤45y	1.14 (0.68-1.90)	1.49 (0.88-2.51)	0.70 / 0.36
				Pre, ≤45y	0.90 (0.51-1.62)	1.49 (0.84-2.65)	0.85 / 0.49
Baglietto, 2007 (48)	coh	Australia	151	Pre	0.83 (0.49-1.38)	0.73 (0.42-1.26)	0.29 / 0.20
			52	<50y	0.60 (0.25-1.45)	0.79 (0.34-1.83)	0.11 / 0.35

[a] Pre = premenopausal, post = postmenopausal; [b] Mutually adjusted for IGFBP-3 and IGF-I, respectively; [c] Adjusted for functional IGFBP-3; [d] Extension of Toniolo, 2000; [e] Update of Hankinson, 1998. References can be found in the references section of Chapter 1.

Appendix 2. Summary of prospective cohort (nested case-control) studies (*coh*) and case-control (*cc*) studies (including at least 100 cancer cases) on circulating IGF-I and IGFBP-3 concentrations and postmenopausal breast cancer risk

Reference	Study design	Country	Cases / controls	Menopausal status[a]	IGF-I Adjusted RR high vs. low (95% CI)	IGFBP-3 Adjusted RR high vs. low (95% CI)	P for trend IGF-I / IGFBP-3
Hankinson, 1998 (28)	coh	US	305 / 483	Post	0.85 (0.93-1.39)	-	0.63
				Post[b]	0.89 (0.51-1.55)	-	0.91
Toniolo, 2000 (29)	coh	US	115 / 220	Post	0.95 (0.49-1.86)	1.08 (0.54-2.16)	0.87 / 0.83
Kaaks, 2002 (30)	coh	Sweden	274 / 519	≥55y	1.29 (0.80-2.07)	1.46 (0.92-2.32)	0.15 / 0.30
Krajcik, 2002 (31)	coh	US	60 / 60	Post	0.77 (0.23-2.56)	-	0.07 / -
				Post[b]	1.22 (0.21-6.78)	0.32 (0.07-1.41)	0.74 / 0.09
Muti, 2002 (32)	coh	Italy	64 / 238	Post	0.58 (0.24-1.36)	0.73 (0.30-1.74)	0.25 / 0.53
Yu, 2002 (33)	cc	China	122 / 126	Post	1.97 (0.93-4.19)	2.60 (1.03-6.56)	0.04 / 0.04
				Post[b]	1.56 (0.68-3.57)	2.11 (0.76-5.87)	0.17 / 0.18
Keinan-Boker, 2003 (34)	coh	The Netherlands	149 / 333	Post	1.1 (0.6-2.1)	1.6 (0.7-3.5)	-
				Post[b]	0.7 (0.3-1.5)	1.4 (0.6-3.4)	-
Gronbaek, 2004 (54)	coh	Denmark	411 / 397	Post	0.97 (0.87-1.08)	1.13 (1.02-1.26)	-
Schairer, 2004 (35)	cc	US	185 / 159	Post[b]	0.9 (0.4–2.0)	1.2 (0.6–2.8)	0.56 / 0.59
			186 epithelial hyperplasia / 159	Post	1.0 (0.4–2.2)	1.2 (0.6–2.8)	0.87 / 0.80
Allen, 2005 (42)	coh	UK	47 / 141	Post	0.77 (0.34-1.74)	0.99 (0.40-2.46)	0.56 / 0.98
				Post[b]	0.73 (0.29-1.84)	1.14 (0.40-3.23)	0.52 / 0.81
Schernhammer, 2005[c] (44)	coh	US	514 / 754	Post	1.0 (0.7-1.4)	0.8 (0.6-1.1)	0.59 / 0.54
Rinaldi, 2006 (45)	coh	Europe	809 / 1564	>50y	1.38 (1.02-1.86)	1.44 (1.04-1.98)	0.01 / 0.01
			524 / 1015	>50y, ≥2y diag	1.45 (1.12-1.87)	1.45 (1.03-2.04)	-
Rollison, 2006 (47)	coh	US	152 / 152	Pre, post	1.36 (0.77-2.39)	0.85 (0.48-1.54)	0.32 / 0.82
				Pre, post[b]	1.60 (0.85-3.02)	0.69 (0.36-1.34)	0.30 / 0.73
			91 / 91	Post, post	1.73 (0.82-3.64)	1.51 (0.75-3.06)	0.13 / 0.11
				Post, post[b]	1.55 (0.61-3.94)	1.17 (0.48-2.84)	0.48 / 0.36
Baglietto, 2007 (48)	coh	Australia	220	Post	1.59 (1.03-2.44)	1.42 (0.92-2.19)	0.05 / 0.06
			50	≥60y	1.61 (1.04-2.51)	1.62 (1.03-2.55)	0.06 / 0.02

[a] Pre = premenopausal, post = postmenopausal; [b] Mutually adjusted for IGFBP-3 and IGF-I, respectively; [c] Update of Hankinson, 1998. References can be found in the references section of Chapter 1.

Appendix 3. Summary of prospective cohort (nested case-control) studies (*coh*) and case-control (*cc*) studies (including at least 100 cancer cases) on circulating IGF-I and IGFBP-3 concentrations and colorectal cancer risk

Reference	Study design	Country (gender)	Cases / controls	Location[a]	IGF-I Adjusted RR high vs. low (95% CI)	IGFBP-3 Adjusted RR high vs. low (95% CI)	P for trend IGF-I / IGFBP-3
Ma, 1999 (36)	coh	US (M)	193 / 318	CR	1.36 (0.72-2.55)	0.47 (0.23-0.95)	0.51 / 0.07
				CR[b]	2.51 (1.15-5.46)	0.28 (0.12-0.68)	0.02 / 0.005
Giovannucci, 2000 (37)	coh	US (F)	79 / 158	CR	1.21 (0.52-2.81)	0.53 (0.18-1.53)	
				CR[b]	2.18 (0.94-5.08)	0.28 (0.10-0.83)	0.10 / 0.05
Kaaks, 2000 (38)	coh	US (F)	102 / 200 (75C / 27R)	CR	1.88 (0.72-4.91)	2.46 (1.09-5.57)	0.25 / 0.19
				CR[b]	1.23 (0.47-3.22)	1.23 (0.51-2.95)	0.70 / 0.51
				C	1.68 (0.58-4.89)	2.19 (0.86-5.59)	0.73 / 0.37
				C[b]	1.36 (0.45-4.11)	1.28 (0.46-3.55)	0.96 / 0.65
Probst-Hensch, 2001 (39)	coh	China (M)	135 / 661	CR	1.52 (0.82-2.85)	1.72 (0.91-3.25)	0.34 / 0.13
				CR[b]	1.18 (0.55-2.53)	1.78 (0.86-3.70)	0.96 / 0.16
Palmqvist, 2002 (40)	coh	Sweden (M,F)	168 / 336 (110C / 58R)	CR	1.27 (0.65-2.47)	1.23 (0.68-2.22)	0.51 / 0.24
				CR[b]	1.27 (0.62-1.63)	1.32 (0.66-1.67)	0.56 / 0.20
				C	2.66 (1.09-6.50)	1.93 (0.92-4.06)	0.03 / 0.02
				C[b]	2.47 (0.93-6.53)	1.75 (0.72-4.22)	0.08 / 0.07
				R	0.33 (0.09-1.13)	0.49 (0.16-1.49)	0.09 / 0.21
				R[2]	0.43 (0.11-1.59)	0.58 (0.16-2.16)	0.23 / 0.45
Nomura, 2003 (41)	coh	US (Japanese-American) (M)	282 / 282 (177C / 105R)	CR[b]	1.5 (0.8-2.8)	0.8 (0.4-1.6)	0.13 / 0.45
				C[b]	1.8 (0.8-4.3)	0.9 (0.5-1.5)	0.12 / 0.60
				R[b]	0.6 (0.2-1.6)	0.9 (0.4-2.0)	0.32 / 0.34
Wei, 2005 (49)	coh	US (F)	182 / 350 (137C / 262contr)	CR	-	-	0.07 / 0.09
				C	1.95 (0.97-3.91)	1.20 (0.62-2.30)	0.09 / 0.62
				C[b]	2.17 (0.96-4.88)	0.81 (0.38-1.75)	0.03 / 0.12
Otani, 2007 (50)[c]	coh	Japan (M,F)	375 / 750 (256C / 119R)	CR	0.8 (0.4-1.7)	1.4 (0.7-2.8)	0.91 / 0.60
				C	0.8 (0.4-1.7)	1.6 (0.7-3.7)	0.89 / 0.41
				R	0.8 (0.4-1.7)	0.9 (0.2-4.2)	0.92 / 0.82

[a]CR = colorectum, C = colon, R = rectum; [b] Mutually adjusted for IGFBP-3 and IGF-I, respectively; [c] Results for men, similar results for women. References can be found in the references section of Chapter 1.

Appendix 4. Summary of prospective cohort (nested case-control) studies (*coh*) on circulating IGF-II concentrations and breast and colorectal cancer risk

Reference	Study design	Country (gender)	Cases / controls	Menopausal status / location[a]	Adjusted RR high vs. low (95% CI)	P for trend
Breast cancer						
Gronbaek, 2004 (54)	coh	Denmark	411 / 397	Post[b]	1.19 (0.98-1.44)	-
Allen, 2005 (42)	coh	UK	69 / 209	Pre	0.86 (0.42-1.76)	0.68
				Pre[c]	1.17 (0.48–2.84)	0.70
			47 / 141	Post	0.87 (0.37–2.05)	0.67
				Post[c]	0.81 (0.29–2.28)	0.63
Colorectal cancer						
Ma, 1999 (36)	coh	US (M)	193 / 318	CR	no association	-
				CR[c]	no association	-
Probst-Hensch, 2001 (39)	coh	China (M)	135 / 661	CR	2.20 (1.15-4.20)	0.03
				CR[c]	2.04 (0.96-4.33)	0.14
Hunt, 2002 (56)	coh	US (F)	102 / 200 (75C / 27R)	CR	2.02 (0.83-4.93)	0.07
				CR[c]	2.07 (0.57-7.88)	0.25
				C	2.02 (0.73-5.61)	0.24
				C[c]	1.77 (0.37-8.44)	0.67
Morris, 2006 (53)	coh	UK (M)	147 / 440	CR	1.59 (0.67-3.75)	0.40

[a] Pre = premenopausal, post = postmenopausal, CR = colorectum, C = colon; [b] Adjusted for IGFBP-2; [c] Adjusted for IGFBP-3. References can be found in the references section of Chapter 1.

Appendix 5. Summary of prospective cohort (nested case-control) studies (*coh*) on circulating IGFBP-1 and IGFBP-2 concentrations and breast and colorectal cancer risk

Reference	Study design	Country	Cases / controls	Menopausal status / location[a]	IGFBP-1 Adjusted RR high vs. low (95% CI)	IGFBP-2 Adjusted RR high vs. low (95% CI)	P for trend IGFBP-1 / -BP-2
Breast cancer							
Kaaks, 2002 (30)	coh	Sweden	130 / 233	Pre + post	1.65 (0.90-3.02)	1.09 (0.57-2.09)	0.17 / 0.97
Krajcik, 2002 (31)	coh	US	66 / 66	Pre	2.40 (0.61–9.51)	1.10 (0.30–4.07)	0.18 / 0.69
			60 / 60	Post	1.96 (0.35–10.9)	0.11 (0.02–0.66)	0.75 / 0.002
Muti, 2002 (32)[b]	coh	Italy	69 / 265	Pre	0.96 (0.39–2.38)	0.66 (0.26–1.64)	0.76 / 0.48
			64 / 238	Post	1.70 (0.70–4.15)	0.87 (0.39–1.92)	0.50 / 0.36
Gronbaek, 2004 (54)	coh	Denmark	411 / 397	Pre	-	1.03 (0.84-1.25)	-
				Post	-	-	-
Schernhammer, 2005 (44)[b]	coh	US	218 / 281	Pre	1.5 (0.8-2.8)	-	0.34
			514 / 754	Post	0.9 (0.6-1.5)		0.25
Schernhammer, 2006 (46)[b]	coh	US	191 / 378	All	0.95 (0.63-1.41)	-	0.51
			129 / 255	Pre	0.68 (0.37-1.24)		0.50
Colorectal cancer							
Kaaks, 2000 (38)	coh	US	102 / 200	CR	0.48 (0.23-1.00)	0.38 (0.15-0.94)	0.02 / 0.06
		(F)	(75C / 27R)	C	0.69 (0.31-1.56)	0.31 (0.11-0.87)	0.20 / 0.09
Palmqvist, 2003 (40)	coh	Sweden	168 / 336	CR	0.68 (0.36-1.29)	0.71 (0.37-1.39)	0.34 / 0.48
		(M, F)	(110C / 58R)	C	0.71 (0.30-1.67)	0.83 (0.36-1.90)	0.46 / 0.75
				R	0.43 (0.14-1.37)	0.38 (0.11-1.37)	0.38 / 0.24
Saydah, 2003 (57)	coh	US	173 / 346	CR	0.93 (0.55-1.58)	-	0.53
		(M, F)	(132C / 41R)	C	no ass		-
Wei, 2005 (49)[b]	coh	US	137 / 262	CR	0.48 (0.21-1.09)	-	0.31
		(M, F)		C	0.28 (0.11-0.75)		0.05
Jenab, 2007 (58)	coh	EU	1,078 / 1,078	CR	0.89 (0.65-1.22)	1.18 (0.86-1.63)	0.53 / 0.17
		(M, F)	(674C / 404R)	C	0.82 (0.55-1.24)	1.48 (0.97-2.24)	0.56 / 0.09
				R	1.01 (0.60-1.68)	0.83 (0.50-1.39)	0.42 / 0.83
Otani, 2007 (50)	coh	Japan	375 / 750	CR	1.1 (0.5-2.5)	-	0.84
		(M, F)	(256C / 119R)	C	1.6 (0.6-4.0)		0.47
				R	0.3 (0.1-1.7)		0.06

[a] Pre = premenopausal, post = postmenopausal, CR = colorectum, C = colon, R = rectum; [b] Only these four studies were conducted under optimal fasting conditions, i.e. fasting for ≥6 h. References can be found in the references section of Chapter 1.

Appendix 6. IGF-I and IGF-II expression in human colorectal (cancer) tissues

Reference	N[a]	Technique[b]	IGF-I expression[c]			IGF-II expression[c]		
			Tumor	*Normal*	*Difference T vs. N*	*Tumor*	*Normal*	*Difference T vs. N*
Tricoli, 1986 (88)	20 T, 20 ANCT	Dot blot	+	very low	↑, 20%	+, 40%	very low	↑
Lambert, 1990 (89)	21 T, 21 ANCT	Dot blot	+	+	=	+, 30%	very low	↑
Michell, 1997 (90)	10 T, 10 ANCT	Dot blot	100%	100%	n.t.	30%	-	↑
Michell, 1997 (90)	10 T, 10 ANCT	IHC	-	-	n.t.	-	-	n.t.
Wang, 1997 (71)	17 T, 17 ANCT	IHC	n.t.	n.t.	n.t.	65%	25%	↑
Kawamoto, 1998 (72)	92 T, 38 ANCT	IHC	n.t.	n.t.	n.t.	74%	11%	↑
Freier, 1999 (81)	6 T, 6 ANCT	RNAse prot	-	-	n.t.	+	+	↑
Freier, 1999 (81)	10 T, 10 ANCT	RIA	+	+	=	+	+	↑
Winkler, 1999 (91)	6 T, 6 ANCT	RIA	n.t.	n.t.	n.t.	+/-	+	↑
Bustin, 2002 (69)	50 T, 50 ANCT	PCR[d]	54%	60%	=	n.t.	n.t.	n.t.
Bustin, 2002 (69)	50 T, 50 ANCT	IHC	+ / -	+ / -	n.t.	n.t.	n.t.	n.t.
Barozzi, 2002 (92)	49 T	IHC	n.t.	n.t.	n.t.	6%	n.t.	n.t.
Weber, 2002 (67)	40 T, 40 ANCT	PCR[e]	n.t.	n.t.	n.t.	+	+	↑, 70%
Peters, 2003 (83)	713 T	IHC	7.5%	n.t.	n.t.	13%	n.t.	n.t.
Li, 2004 (73)	48 T, 48 ANCT	PCR[d]	n.t.	n.t.	n.t.	+	+	↑
Nosho, 2004 (68)	27 T	PCR[f]	81%	very low	↑	70%	+	↑
Nosho, 2004 (68)	60 T	IHC	n.t.	n.t.	n.t.	58%	n.t.	n.t.
Jenkins, 2005 (70)	46 T, 46 ANCT	PCR[d]	41%	67%	=	n.t.	n.t.	n.t.
Nosho, 2005 (93)	14 T, 14 ANCT	cDNA array	n.t.	n.t.	n.t.			↑

[a] T = colorectal tumor tissue, ANCT = adjacent normal colorectal tissue; [b] Dot blot = RNA dot blotting, IHC = immunohistochemistry, RNAse prot = RNAse protection assay, RIA = radioimmunoassay, PCR = polymerase chain reaction; [c] + = positive, - = negative, '=' = no difference, n.t. = not tested, ↑ = increased; [d] real-time RT-PCR; [e] competitive RT-PCR (MIMIC); [f] semiquantitative RT-PCR.

Appendix 7. IGF-IR expression in human colorectal (cancer) tissues

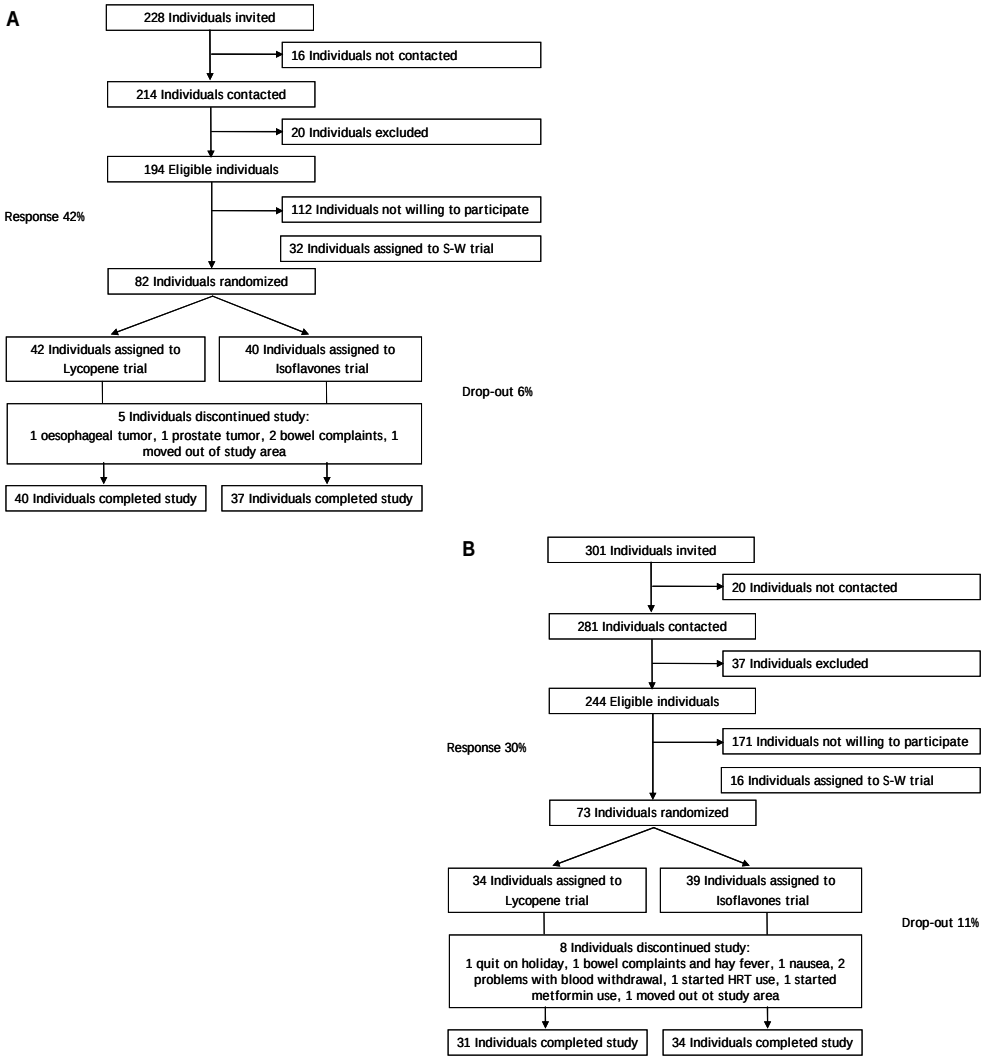
Reference	*N*[a]	Technique[b]	Tumor[c]	Normal[c]	Difference T vs. N[c]
Pollak, 1987 (74)	15 T, 15 ANCT	IGF binding	+	+	=
Rouyer-Fessard, 1990 (75)	9 ANCT	IGF binding	n.t.	+	n.t.
Guo, 1992 (76)	3 T	IGF binding	+	n.t.	n.t.
Adenis, 1995 (77)	20 T, 20 ANCT	IGF binding	70%	46%	=
Bhatavdekar, 1995 (78)	59 T	IGF binding	15%	n.t.	n.t.
Zenilman, 1997 (79)	4 T, 4 ANCT	PCR[d]	+	+	=
Mishra, 1998 (80)	9 T, 9 ANCT	RNAse prot	+	+	=
Freier, 1999 (81)	6 T, 6 ANCT	RNAse prot	+	+	↑
Hakam, 1999 (65)	36 T, 34 ANCT	IHC	96%	-	↑
Bustin, 2002 (69)	50 T, 50 ANCT	PCR[e]	100%	100%	=
Weber, 2002 (67)	40 T, 40 ANCT	PCR[f]	100%	100%	↑, 80%
Weber, 2002 (67)	18 T, 18 ANCT	IGF binding	+	+	↑
Weber, 2002 (67)	33 T, 33 ANCT	IHC	91%	very low	↑, 91%
Ouban, 2003 (82)	10 T, 10 ANCT	IHC	90%	-	↑
Peters, 2003 (83)	713 T	IHC	99%	n.t.	n.t.
Koda, 2004 (84)	144 T	IHC	51%	n.t.	n.t.
Nakamura, 2004 (85)	161 T	IHC	98%	n.t.	n.t.
Nosho, 2004 (68)	27 T	PCR[g]	48%	very low	↑
Jenkins, 2005 (70)	46 T, 46 ANCT	PCR[e]	100%	100%	=
Cunningham, 2006 (86)	87 T	IHC	93%	n.t.	n.t.
Sulkowski, 2006 (87)	115 T	IHC	47%	n.t.	n.t.

[a] T = colorectal tumor tissue, ANCT = adjacent normal colorectal tissue; [b] IGF binding = radioligand ([^{125}I]IGF-I) binding assay, PCR = polymerase chain reaction, RNAse prot = RNAse protection assay, IHC = immunohistochemistry; [c] '+' = positive, '-' = negative, '=' = no difference, n.t. = not tested, '↑' = increased; [d] quantitative RD-PCR; [e] real-time RT-PCR; [f] competitive RT-PCR (MIMIC); [g] semiquantitative RT-PCR. References can be found in the references section of Chapter 1.

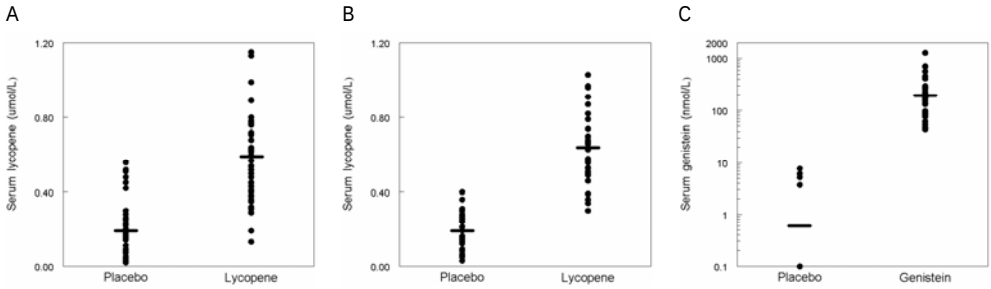
Appendix 8. Response rates, number of participants in the study and total time contributed to the study per hospital for the population at increased colorectal cancer risk

	Response (%)			Number of participants in study			Time
Hospital	**Total**	**Men**	**Women**	**Total**	**Men**	**Women**	**(mo)**
1	29	29	29	17 (11%)	9 (11%)	8 (11%)	27
2	38	52	28	64 (41%)	38 (46%)	26 (36%)	27
3	33	48	29	30 (19%)	10 (12%)	20 (27%)	11
4	36	36	36	44 (28%)	25 (31%)	19 (26%)	22
Total	**35**	**42**	**30**	**155**	**82**	**73**	-

Appendix 9. Flow charts for men (A) and women (B), for the lycopene and isoflavone intervention trials combined



Appendix 10. Serum lycopene (A in men, B in women) and genistein (C in men) concentrations after placebo and after lycopene or isoflavone supplementation



Summary

This thesis addresses the effects of dietary interventions with lycopene and isoflavone supplementation on the circulating insulin-like growth factor (IGF) system, and also describes molecular studies in which the role of the IGF-system in breast and colorectal carcinogenesis is investigated. The IGF-system is primarily involved in the regulation of prenatal and postnatal growth, and IGF-system components are expressed in most tissues. However, the liver is the principal source for the two ligands IGF-I and IGF-II in the blood circulation. About 90% of IGF-I in the circulation is bound to both IGF binding protein -3 (IGFBP-3) and acid labile subunit. This complex is too large to pass the capillary epithelium, resulting in an increased half-life of IGF-I. Free IGF-I (<1%) and IGF-I bound to IGFBP-1 or IGFBP-2 can be transported out of the bloodstream to specific target tissues. IGFBPs are degradable by proteases, rendering IGF-I free to interact with the IGF-I receptor (IGF-IR). Binding of IGF-I or IGF-II to the IGF-IR results in receptor phosphorylation, activation of downstream targets, and stimulation of proliferation and inhibition of apoptosis.

Epidemiological studies indicate that high circulating IGF-I concentrations are associated with increased risk of cancer, in particular risk of premenopausal breast cancer and colorectal cancer (**Chapters 1 and 2**). High circulating IGF-II concentrations have also been associated with increased colorectal cancer risk. Qualitative and semi-quantitative studies have frequently shown overexpression of the IGF-IR in breast tumor and colorectal tumor tissues. However, results from quantitative studies are scarce and inconsistent. Circulating concentrations of IGF-I are determined by genetic factors as well as dietary and lifestyle factors. *In vitro* and *in vi*vo studies and epidemiological studies have shown that the dietary factors lycopene and isoflavones may decrease circulating IGF-I concentrations and increase circulating IGFBP-3 concentrations. Lycopene is a carotenoid primarily present in tomatoes and tomato products. Isoflavones have structural and functional similarities to estrogens, are mainly present in soy foods, but can also be derived from red clover. The effects of lycopene and isoflavones on the IGF-system in humans are tested most optimally in intervention trials using dietary supplements.

The main aim of the studies described in this thesis was to investigate the effects of lycopene and isoflavone supplementation on circulating IGF-I and other IGF-system components in premenopausal women at increased breast cancer risk, and in men and women at increased colorectal cancer risk. Additionally, we examined whether differences exist in expression levels of IGF-system components between normal breast and breast tumor tissues, and we tried to elucidate the relation between levels of IGF-system components in normal colorectal tissue and serum.

Cross-sectional study

We first investigated whether habitual dietary intake of lycopene and tomatoes, phytoestrogens (including isoflavones) and related foods, total energy, protein, and alcohol was associated with plasma levels of IGF-I and IGFBP-1, IGFBP-2, and IGFBP-3 (**Chapter 3**). Therefore, a cross-sectional study was conducted in 224 premenopausal and

162 postmenopausal healthy Dutch women, aged 49-69 years, participating in the Prospect-EPIC study in the Netherlands. Diet was assessed using a food frequency questionnaire. In this study, no independent associations of dietary factors with IGF-I or IGFBP-3 concentrations were observed. Among the lycopene and isoflavone containing foods investigated, only an association between increased intake of soy products and higher plasma IGFBP-2 concentrations in premenopausal women was found (p = 0.04). The habitual dietary intake of lycopene and isoflavones in this study population was low (median intake ~3 mg/day and 0.15 mg/day, respectively). Apparently, this low intake was not associated with circulating total IGF-I and IGFBP-1, IGFBP-2, and IGFBP-3 concentrations.

Dietary intervention studies

To accurately examine whether higher intake of lycopene and isoflavones affects circulating IGF-system components, we conducted randomized, placebo-controlled, double-blind cross-over studies with tomato-derived lycopene (30 mg/day) and red clover-derived isoflavone (84 mg/day) supplementation. The total duration of the studies was approximately 6 months, consisting of two 2-month intervention periods separated by a 2-month washout period. Most of the men and women at increased colorectal cancer risk underwent a colonoscopy at the end of the first intervention period. The main parameter of interest was the relative cross-over difference in serum total IGF-I concentrations (i.e., concentration after intervention minus concentration after placebo treatment, expressed as percentage change relative to the concentration after placebo treatment). Relative cross-over differences in serum concentrations of free IGF-I, IGF-II, IGFBP-1, IGFBP-2, and IGFBP-3 were secondary endpoints.

Lycopene and IGF

In **Chapter 4**, we evaluated the effect of lycopene supplementation in premenopausal women with (1) a history of breast cancer (n = 24), or (2) a high familial breast cancer risk (n = 36), ≤50 years of age. Lycopene supplementation did neither significantly alter serum total IGF-I nor any of the other IGF-system components in the two study populations combined. However, statistically significantly discordant results were observed between the two study populations (i.e., $p < 0.05$ for total IGF-I, free IGF-I and IGFBP-3). Total IGF-I and IGFBP-3 were increased in the breast cancer survivor population (mean relative difference between serum total IGF-I concentrations after lycopene supplementation and after placebo, 7.0%, 95%CI -0.2% to 14.3%; IGFBP-3, 3.3%, 95%CI 0.7% to 6.0%), and free IGF-I was decreased in women with a family history of breast cancer (-7.6%, 95%CI -14.6% to -0.6%). No changes were observed in circulating concentrations of IGFBP-1 and IGFBP-2. These results suggest an IGF-I lowering effect of lycopene only in healthy women with a family history of breast cancer, not in breast cancer survivors. This may be due to interference of genetic background or disease status with lycopene effects on the IGF-system. As the IGF-system is known to interact with sex steroid hormone pathways, any

effects of dietary factors on the IGF-system may also differ by gender and menopausal status. However, these results may also be due to chance.

We also investigated the effect of lycopene supplementation in a similar study in 40 men (aged 40-75 years) and 31 postmenopausal women (aged 50-75 years) with a family history of colorectal cancer and/or a personal history of colorectal adenomas (**Chapter 5**). In both men and women, lycopene supplementation did not significantly affect serum total IGF-I concentrations. Hoever, lycopene supplementation significantly increased serum IGFBP-1 concentrations in women (median relative difference 21.7%, p = 0.01). Serum IGFBP-2 concentrations were increased in both men and women after lycopene supplementation, but to a lesser extent than IGFBP-1 (mean relative difference 8.2%, 95%CI 0.7% to 15.6%; and 7.8%, 95%CI -5.0% to 20.6%, respectively). IGF-II and IGFBP-3 concentrations were not markedly altered, and free IGF-I concentrations were not measured. Although lycopene supplementation did not influence serum total IGF-I concentrations in men and women at increased colorectal cancer risk, our results indicate that it may decrease IGF-I bioavailability by increasing IGFBP-1 and IGFBP-2 concentrations. However, we were the first to investigate lycopene effects on circulating concentrations of IGFBP-1 and IGFBP-2, and results need to be confirmed in larger randomized intervention studies.

Isoflavones and IGF

The effect of isoflavone supplementation on circulating IGF-system components was investigated in men (aged 40-75 years) and postmenopausal women (aged 50-75 years) with a family history of colorectal cancer and/or a personal history of colorectal adenomas. In 37 men, isoflavone supplementation did not significantly affect serum total IGF-I concentrations (mean relative difference -1.3%, 95%CI -8.6% to 6.0%), or any of the other IGF-system components (**Chapter 6**). Previous studies have shown that about 30-50% of individuals are able to convert daidzein, one of the main isoflavone metabolites, to the more potent estrogenic metabolite equol. Interestingly, in our study higher serum concentrations of equol were associated with decreases in serum IGF-I concentrations after isoflavone supplementation (r = -0.49, p = 0.002). In conclusion, isoflavone supplementation did not affect circulating concentrations IGF-system components in men at increased colorectal cancer risk. However, to our knowledge, this is the first study that suggests isoflavones might have an IGF-I lowering effect in equol producers only. This underlines the importance of taking into account equol status in future isoflavone intervention studies.

Similarly, isoflavone supplementation did not significantly affect serum concentrations of total IGF-I in 34 postmenopausal women (mean relative difference -2.0%, 95%CI -8.0% to 3.9%) (**Chapter 7**). Neither IGF-II nor IGFBPs were significantly altered after isoflavone supplementation. Moreover, we observed no differences in mRNA expression levels of IGF-system components in normal colorectal tissue biopsies between women on isoflavones and women on placebo. These results suggest that the increased serum IGF-I concentrations observed in previous studies investigating soy food or soy protein

supplementation are most likely due to soy protein itself, and not to isoflavones.

Molecular studies

Breast carcinogenesis

Protein and mRNA expression of IGF-system components in human breast tissue have typically been studied using qualitative or semi-quantitative techniques. Results with respect to mRNA expression in normal breast and breast tumor tissue are inconsistent and inconclusive, and quantitative data on mRNA expression in different types of human breast tissue are lacking. To investigate the plausible causative link between susceptibility (i.e., high serum IGF-I levels and cancer susceptibility) and tumor induction and promotion (i.e., tissue expression and subsequent cancer risk), quantitative data on mRNA expression levels of IGF-system components in breast tissue are essential. In **Chapter 8**, we quantitatively assessed mRNA expression of IGF-I, IGF-II, and their receptors (IGF-IR and IGF-IIR) in breast tissue samples (n = 83) from 72 women by real-time RT-PCR. We evaluated whether mRNA expression levels differ in both normal and tumor breast tissue of women with and without a family history of breast cancer. We found a large variation in mRNA levels. Expression of each gene was significantly higher in normal tissue than in tumor tissue (median for normal and tumor tissue, respectively (arbitrary units); IGF-I: 25.2 and 1.4; IGF-II: 5.9 and 0.6; IGF-IR: 0.18 and 0.07; IGF-IIR: 1.8 and 0.9; $p < 0.0001$). Interestingly, in tumor tissue from patients with a strong family history of breast cancer, expression of both receptors was higher than in tumor tissue from sporadic patients (IGF-IR: 0.13 and 0.05, p = 0.04; IGF-IIR: 1.1 and 0.8, p = 0.04). For cancer-free controls, expression of IGF-II and IGF-IIR in normal breast tissue was also higher in women with a family history of breast cancer than in women without such a family history (IGF-II: 7.2 and 1.5, p = 0.02; IGF-IIR: 2.6 and 1.5, p = 0.09). Our study quantitatively shows that mRNA expression levels of IGF-system components in the breast are generally higher in normal tissue compared with tumor tissue, and higher in tissue from women with a family history of breast cancer than in tissue from women without such a family history. A basis has therefore been created for studies aimed at understanding IGF as a breast cancer risk factor, the relationship between IGF-systems in serum and tissues, and effects of lifestyle factors on the IGF-system.

Colorectal carcinogenesis

In human colorectal tumors, studies using qualitative or semi-quantitative techniques have shown that the IGF-IR and particularly IGF-II are frequently overexpressed compared with normal colorectal tissue. At present, no quantitative data are available on mRNA expression levels of IGF-system components in different locations of the colon. Moreover, it is unknown whether circulating IGF-I and IGF-II proteins directly affect colorectal tumor growth in humans through IGF-IR binding and activation, whether they influence local tissue expression of IGF-system components (e.g. upregulation of IGF-I, IGF-II, or IGF-IR), or whether they are reflective of tissue IGF-system component expression and thereby act

as a biomarker of tissue IGF-system component bioactivity. To investigate this in more depth, biopsies from macroscopically normal mucosa at four locations in the colorectum (ascending, transverse, and sigmoid colon, rectum) and a fasting serum sample were obtained from 48 asymptomatic patients at increased colorectal cancer risk (**Chapter 9**). We quantitatively evaluated mRNA expression levels of IGF-I, IGF-II, IGF-IR, IGF-IIR, and IGFBP-3 by real-time RT-PCR. Expression of IGF-IR protein in the ascending colon and rectum tissue specimens was assessed semi-quantitatively by immunohistochemistry. Additionally, we studied the relationship of tissue mRNA and protein expression with serum IGF-I and IGF-II concentrations. With the exception of IGF-IIR, mRNA levels of all the IGF-system components investigated, as well as IGF-IR protein expression, were significantly higher in the rectum compared with the ascending colon ($p \leq 0.001$). Circulating IGF-I and IGF-II concentrations did not correlate with any of the parameters studied in colorectal tissues. Our results indicate that in humans IGF-system components are differentially expressed in the colorectum. Moreover, our findings suggest that local and circulating components of the IGF-system are differentially regulated. Our data underline the importance of taking into account the colorectal location when investigating dietary or pharmacological effects on colorectal tissue mRNA expression of IGF-system components.

Conclusions

In **Chapter 10** the findings of our studies are summarized, discussed, and integrated. In our randomized controlled cross-over studies, lycopene did not decrease circulating IGF-I concentrations in women at increased premenopausal breast cancer risk or in men and women at increased colorectal cancer risk. Isoflavones did not decrease circulating IGF-I concentrations in men and women at increased colorectal cancer risk either. These results are in line with results from previous studies. Interestingly, lycopene increased IGFBP-1 concentrations in women and IGFBP-2 concentrations in men and in women at increased colorectal cancer risk, thereby possibly decreasing bioavailable IGF-I. Additionally, our results suggest that isoflavones may decrease circulating IGF-I concentrations in equol producers only, which constitute about 30-50% of the Western population. Both findings need further investigation in future randomized controlled trials. The results of our studies and other evidence published to date do not provide support for health claims for lycopene and isoflavones in lowering circulating IGF-I concentrations in women at increased risk of premenopausal breast cancer and men and women at increased risk of colorectal cancer. Although both circulating and local tissue IGF-system components have been related to breast and colorectal carcinogenesis, we did not find an association between colorectal tissue and circulating levels of these components. Whether circulating IGF-I is causally related to cancer risk in humans needs further investigation.

Samenvatting

Dit proefschrift richt zich op de effecten van voedingsinterventies met lycopeen- en isoflavonensuppletie op het insuline-achtige groeifactor (IGF) systeem en beschrijft daarnaast moleculaire studies waarin de rol van het IGF-systeem bij het ontstaan van borst- en dikkedarmkanker wordt onderzocht. Het IGF-systeem is voornamelijk betrokken bij de regulatie van normale groei, en componenten van het IGF-systeem komen tot expressie in de meeste weefsels. De lever is echter de belangrijkste bron voor de twee liganden IGF-I en IGF-II in de bloedcirculatie. Ongeveer 90% van het IGF-I in de circulatie is gebonden aan het IGF bindingseiwit -3 (IGFBP-3) en een zuur-labiele subunit. Dit complex is te groot om de bloedbaan te kunnen passeren, hetgeen resulteert in een toegenomen halfwaardetijd van IGF-I. Vrij IGF-I (<1%) en IGF-I gebonden aan IGFBP-1 en IGFBP-2 kan uit de bloedbaan getransporteerd worden naar specifieke weefsels. IGFBPs kunnen worden afgebroken door proteases, waardoor het vrijgekomen IGF-I kan reageren met de IGF-I receptor (IGF-IR). Binding van IGF-I of IGF-II aan de IGF-IR resulteert in fosforylatie van de receptor, activatie van downstream moleculen, en stimulatie van celgroei en remming van celdood.

Epidemiologische studies laten zien dat hoge concentraties circulerend IGF-I geassocieerd zijn met een verhoogd risico op kanker, met name het risico op premenopauzale borstkanker en dikkedarmkanker (**Hoofdstuk 1 en 2**). Hoge circulerende IGF-II concentraties zijn ook geassocieerd met een verhoogd risico op dikkedarmkanker. Kwalitatieve en semikwantitatieve studies hebben vaak een verhoogde expressie van de IGF-IR gevonden in borsttumorweefsels en in dikkedarmtumorweefsels. De resultaten van kwantitatieve studies zijn echter schaars en inconsistent. Circulerende concentraties van IGF-I worden bepaald door zowel genetische als voedings- en leefstijlfactoren. Studies in cellen en dieren en epidemiologische studies duiden erop dat de voedingsstoffen lycopeen en isoflavonen circulerende IGF-I concentraties mogelijk zouden kunnen verlagen en circulerende IGFBP-3 concentraties zouden kunnen verhogen. Lycopeen is een carotenoïde die voornamelijk voorkomt in tomaten en tomatenproducten. Isoflavonen lijken in structuur en functie op oestrogenen en komen met name voor in sojaproducten, maar kunnen ook verkregen worden uit rode klaver. De effecten van lycopeen en isoflavonen op het IGF-systeem in mensen kan het meest optimaal getest worden in interventiestudies gebruikmakend van voedingssupplementen.

Het belangrijkste doel van de studies beschreven in dit proefschrift was om de effecten te onderzoeken van suppletie met lycopeen en isoflavonen op circulerende concentraties van IGF-I en andere IGF-systeem componenten bij premenopauzale vrouwen met een verhoogd risico op borstkanker, en bij mannen en vrouwen met een verhoogd risico op dikkedarmkanker. Daarnaast hebben we onderzocht of er verschillen bestaan in expressieniveaus van IGF-systeem componenten tussen normaal borstweefsel en borsttumorweefsel en probeerden we opheldering te verkrijgen over de relatie tussen niveaus van IGF-systeem componenten in normaal dikkedarmweefsel en in bloed.

Crossectionele studie

We hebben eerst onderzocht of de gebruikelijke voedingsinname van lycopeen en tomaten, fyto-oestrogenen (waaronder isoflavonen) en de producten rijk aan deze stoffen, en de inname van de totale hoeveelheid energie, eiwit en alcohol geassocieerd was met plasmaniveaus van IGF-I en IGFBP-1, IGFBP-2 en IGFBP-3 (**Hoofdstuk 3**). Daartoe werd een crossectionele studie uitgevoerd bij 224 premenopauzale en 162 postmenopauzale gezonde Nederlandse vrouwen, 49 tot 69 jaar oud, die deelnamen aan de Prospect-EPIC studie in Nederland. De voedingsinname werd geschat met behulp van een voedselfrequentievragenlijst. In deze studie werden geen onafhankelijke associaties gevonden van voedingsfactoren met IGF-I of IGFBP-3 plasmaconcentraties. Van de voedingsmiddelen die lycopeen en isoflavonen bevatten was alleen een verhoogde inname van sojaproducten geassocieerd met hogere IGFBP-2 plasmaconcentraties (p = 0.04). De gebruikelijke voedingsinname van lycopeen en isoflavonen van deze studiepopulatie was laag (mediane inname respectievelijk 3 mg/dag en 0.15 mg/dag). Blijkbaar was deze lage inname niet geassocieerd met circulerende concentraties van IGF-I en IGFBP-1, IGFBP-2 en IGFBP-3.

Voedingsinterventiestudies

Om nauwkeurig te onderzoeken of een hogere inname van lycopeen en isoflavonen bloedconcentraties van IGF-systeem componenten beïnvloedt, hebben we gerandomiseerde, placebo-gecontroleerde, dubbelblinde crossover studies uitgevoerd met een tomatenextract (30 mg/dag lycopeen, capsules) en een rode klaver extract (84 mg/dag isoflavonen, tabletten). De totale duur van de studies was ongeveer 6 maanden, bestaande uit twee 2 maanden durende interventieperiodes die van elkaar gescheiden werden door een 2 maanden durende wash-out periode. De meeste mannen en vrouwen met een verhoogd risico op dikkedarmkanker ondergingen een dikkedarmonderzoek aan het einde van de eerste interventieperiode. De belangrijkste parameter was het relatieve crossover verschil in serumconcentraties van totaal IGF-I (d.w.z. de concentratie na interventie- minus concentratie na placebo-behandeling, uitgedrukt als een procentuele verandering ten opzichte van de concentratie na placebo-behandeling). Relatieve crossover verschillen in serumconcentraties van vrij IGF-I, IGF-II, IGFBP-1, IGFBP-2 en IGFBP-3 waren secundaire eindpunten.

Lycopeen en IGF

In **Hoofdstuk 4** hebben we het effect van lycopeensuppletie onderzocht bij premenopauzale vrouwen met (1) een persoonlijke geschiedenis van borstkanker (n = 24), of (2) een familiegeschiedenis van borstkanker (n = 36), die hoogstens 50 jaar oud waren. **In** de twee studiepopulaties tezamen had lycopeensuppletie geen effect op serumniveaus van totaal IGF-I, noch op een van de andere IGF-systeem componenten. Tussen de twee studiepopulaties werden echter statistisch significante discordante resultaten gevonden (i.e., $p < 0.05$ voor totaal IGF-I, vrij IGF-I en IGFBP-3). Totaal IGF-I en IGFBP-3 waren

gestegen bij de overlevenden van borstkanker (gemiddelde relatieve verschil tussen serum totaal IGF-I concentraties na lycopeensuppletie en na placebo, 7.0%, 95% btbh-i -0.2% tot 14.3%; IGFBP-3, 3.3%, 95% btbh-i 0.7% tot 6.0%), en vrij IGF-I was afgenomen bij vrouwen met een familiegeschiedenis van borstkanker (-7.6%, 95% btbh-i -14.6% tot -0.6%). Er werden geen veranderingen gevonden in circulerende concentraties van IGFBP-1 en IGFBP-2. Deze resultaten suggereren dat lycopeen alleen een IGF-I verlagend effect heeft bij gezonde vrouwen met een familiegeschiedenis van borstkanker en niet bij overlevenden van borstkanker. Dit kan verklaard worden door interferentie van de genetische achtergrond of ziektestatus met lycopeeneffecten op het IGF-systeem. Aangezien bekend is dat het IGF-systeem interactie aangaat met signaaltransductieroutes van geslachtshormoonsteroïden, zouden effecten van voedingsfactoren ook kunnen verschillen naar geslacht of menopauzale status. Deze resultaten kunnen echter ook een toevalsbevinding zijn.

We onderzochten het effect van lycopeensuppletie eveneens in een vergelijkbare studie bij 40 mannen (40 tot 75 jaar oud) en 31 postmenopauzale vrouwen (50 tot 75 jaar oud) met een familiegeschiedenis van dikkedarmkanker en/of een persoonlijke geschiedenis van adenomateuze poliepen in de dikke darm (**Hoofdstuk 5**). Bij zowel mannen als vrouwen had lycopeensuppletie geen significante invloed op serum totaal IGF-I concentraties. Lycopeensuppletie verhoogde echter serum IGFBP-1 concentraties bij vrouwen (mediane relatieve verschil 21.7%, p = 0.01). Serum IGFBP-2 concentraties na lycopeensuppletie waren toegenomen bij zowel mannen als vrouwen, maar in mindere mate dan de IGFBP-1 concentraties (gemiddelde relatieve verschil respectievelijk 8.2%, 95% bthbh-i 0.7% tot 15.6% en 7.8%, 95% btbh-i -5.0% tot 20.6%). IGF-II en IGFBP-3 concentraties waren niet noemenswaardig veranderd en de vrije IGF-I concentraties waren niet bepaald. Ondanks het feit dat lycopeensuppletie de serum totaal IGF-I concentraties bij mannen en vrouwen met een verhoogd riscio op dikkedarmkanker niet beïnvloedde, duiden onze resultaten erop dat het de IGF-I biobeschikbaarheid zou kunnen verlagen door het verhogen van IGFBP-1 en IGFBP-2 concentraties. We waren echter de eerste die de effecten van lycopeen op circulerende concentraties van IGFBP-1 en IGFBP-2 hebben onderzocht en de resultaten moeten bevestigd worden in grotere gerandomiseerde interventiestudies.

Isoflavonen en IGF

Het effect van isoflavonensuppletie op circulerende concentraties van IGF-systeem componenten werd onderzocht bij 37 mannen (40 tot 75 jaar oud) en 34 postmenopauzale vrouwen (50 tot 75 jaar) met een familiegeschiedenis van dikkedarmkanker en/of een persoonlijke geschiedenis van adenomateuze poliepen in de dikke darm. Bij mannen had isoflavonensuppletie geen effect op serumconcentraties van totaal IGF-I (gemiddelde relatieve verschil -1.3%, 95% btbh-i -8.6% tot 6.0%), noch op een van de andere IGF-systeem componenten (**Hoofdstuk 6**). Eerdere studies hebben laten zien dat ongeveer 30-50% van de mensen in staat is om daidzeïne, een van de belangrijkste

isoflavonenmetabolieten, om te zetten in de meer potente oestrogene metaboliet equol. Interessant is dat in onze studie hogere serumconcentraties van equol geassocieerd waren met verlagingen in serum IGF-I concentraties na isoflavonensuppletie (r = -0.49, p = 0.002). Onze conclusie was dat isoflavonenensuppletie de circulerende IGF-I concentraties niet beïnvloedde bij mannen met een verhoogd risico op dikkedarmkanker. Voor zover ons bekend is dit echter de eerste studie die suggereert dat isoflavonen alleen een IGF-I verlagend effect zouden kunnen hebben bij mannen die equol kunnen vormen. Dit onderschrijft het belang om rekening te houden met de equol status in toekomstige interventiestudies met isoflavonen.
Isoflavonensuppletie had eveneens geen significant effect op serumconcentraties van totaal IGF-I bij postmenopauzale vrouwen (gemiddelde relatieve verschil -2.0%, 95% btbh-i -8.0% tot 3.9%) (**Hoofdstuk 7**). IGF-II noch IGFBPs waren significant veranderd na isoflavonensuppletie. Bovendien vonden we geen verschillen in mRNA expressieniveaus van IGF-systeem componenten in biopten van normaal dikkedarmweefsel tussen vrouwen op isoflavonen en vrouwen op placebo. Deze resultaten suggereren dat de verhoogde serum IGF-I concentraties die gevonden zijn in eerdere studies naar soja- of soja-eiwitsuppletie meest waarschijnlijk veroorzaakt worden door het soja-eiwit zelf, en niet door de isoflavonen.

Moleculaire studies

Borstcarcinogenese
De eiwit- en mRNA expressie van IGF-systeem componenten in humaan borstweefsel zijn voornamelijk onderzocht met gebruikmaking van kwalitatieve of semikwantitatieve technieken. De resultaten met betrekking tot mRNA expressie in normaal borst- en borsttumorweefsel zijn inconsistent en inconclusief, en kwantitatieve gegevens over mRNA expressie in verschillende types humaan borstweefsel ontbreken. Voor het onderzoeken van de aannemelijk oorzakelijke relatie tussen susceptibiliteit (d.w.z. hoge serumniveaus van IGF-I en susceptibiliteit voor kanker) en tumorinductie en –promotie (d.w.z. weefselexpressie en opeenvolgend risico op kanker) zijn kwantitatieve gegevens over mRNA expressie van IGF-systeem componenten in borstweefsel essentieel. In **Hoofdstuk 8** hebben we de mRNA expressie van IGF-I, IGF-II en hun receptoren (IGF-IR en IGF-IIR) kwantitatief bepaald in borstweefselsamples (n = 83) van 72 vrouwen met behulp van real-time RT-PCR. We onderzochten of mRNA expressieniveaus verschillen in zowel normaal borstweefsel als borsttumorweefsel van vrouwen met en zonder een familiegeschiedenis van borstkanker. We vonden een grote variatie in mRNA niveaus. De expressie van elk gen was significant hoger in normaal weefsel dan in tumorweefsel (mediaan voor normaal en tumorweefsel, respectievelijk (arbitraire eenheden); IGF-I: 25.2 en 1.4; IGF-II: 5.9 en 0.6; IGF-IR: 0.18 en 0.07; IGF-IIR: 1.8 en 0.9; $p < 0.0001$). Het was interessant dat in tumorweefsel van patiënten met een sterke familiegeschiedenis van borstkanker de expressie van beide receptoren hoger was dan in tumorweefsel van sporadische patiënten (IGF-IR: 0.13 en 0.05, p = 0.04; IGF-IIR: 1.1 en 0.8, p = 0.04). Voor

controles zonder kanker was de expressie van IGF-II en de IGF-IIR in normaal borstweefsel ook hoger bij vrouwen met een familiegeschiedenis van borstkanker dan bij vrouwen zonder een dergelijke familiegeschiedenis (IGF-II: 7.2 en 1.5, p = 0.02; IGF-IIR: 2.6 en 1.5, p = 0.09). Onze studie laat op kwantitatieve wijze zien dat mRNA expressieniveaus van IGF-systeem componenten in de borst over het algemeen hoger zijn in normaal weefsel in vergelijking met tumorweefsel, en hoger in weefsel van vrouwen met een familiegeschiedenis van borstkanker dan in weefsel van vrouwen zonder een dergelijke familiegeschiedenis. Hiermee is een basis gelegd voor studies die als doel hebben om kennis te verkrijgen over IGF als een risicofactor voor borstkanker, over de relatie tussen het IGF-systeem in serum en weefsels, en de effecten van leefstijlfactoren op het IGF-systeem.

Dikkedarmcarcinogenese

Studies in humane dikkedarmtumoren hebben, gebruikmakend van kwalitatieve of semikwantitatieve technieken, laten zien dat de IGF-IR en met name IGF-II vaak verhoogd tot expressie komen in vergelijking met normaal dikkedarmweefsel. Momenteel zijn geen kwantitatieve gegevens beschikbaar met betrekking tot mRNA expressieniveaus van IGF-systeem componenten in verschillende locaties van de dikke darm. Bovendien is het onbekend of circulerende IGF-I en IGF-II eiwitten rechtstreeks de groei van humane dikkedarmtumoren beïnvloeden door binding en activering van de IGF-IR, of ze van invloed zijn op de locale weefselexpressie van IGF-systeem componenten (bv. opregulatie van IGF-I, IGF-II, of IGF-IR), of dat ze een weerspiegeling zijn van de weefselexpressie van IGF-systeem componenten en daarmee een biomerker zijn van de bioactiviteit van IGF-systeem componenten in weefsel. Om dit meer diepgaand te onderzoeken werden biopten van macroscopisch normaal mucosaal weefsel verkregen op vier locaties in de dikke darm (colon ascendens, colon transversum, sigmoïd, en rectum) van 48 asymptomatische patiënten met een verhoogd risico op dikkedarmkanker (**Hoofdstuk 9**). We hebben op kwantitatieve wijze de mRNA expressieniveaus van IGF-I, IGF-II, IGF-IR, IGF-IIR en IGFBP-3 onderzocht met behulp van real-time RT-PCR. De eiwitexpressie van de IGF-IR in de colon ascendens en het rectum werd semikwantitatief beoordeeld met behulp van immunohistochemie. In aanvulling daarop bestudeerden we de relatie van weefsel mRNA en eiwitexpressie met serum IGF-I en IGF-II concentraties. Met uitzondering van de IGF-IIR waren de mRNA niveaus van alle onderzochte IGF-systeem componenten, evenals de eiwitexpressie van de IGF-IR, significant hoger in het rectum in vergelijking met het colon ascendens ($p \leq 0.001$). Circulerende IGF-I en IGF-II concentraties correleerden met geen enkele van de bestudeerde parameters in dikkedarmweefsel. Onze resultaten wijzen op differentiële expressie van IGF-systeem componenten in de humane dikke darm. Bovendien suggereren onze uitkomsten dat locale en circulerende componenten van het IGF-I systeem differentieel gereguleerd zijn. Onze bevindingen onderschrijven het belang om rekening te houden met de locatie in de dikke darm wanneer voedingseffecten of farmacologische effecten worden onderzocht op mRNA expressie van IGF-systeem

componenten in dikkedarmweefsel.

Conclusies

In **Hoofdstuk 10** worden de uitkomsten van onze studies samengevat, bediscussieerd en geïntergreerd. In onze gerandomiseerde, gecontroleerde crossover studies werd geen verlagend effect gevonden van lycopeen op de circulerende IGF-I concentraties bij vrouwen met een verhoogd risico op premenopauzale borskanker of bij mannen en vrouwen met een verhoogd risico op dikkedarmkanker. Isoflavonen verlaagden evenmin de circulerende IGF-I concentraties bij mannen en vrouwen met een verhoogd risico op dikkedarmkanker. Deze resultaten zijn in lijn met resultaten van eerdere studies. Interessant is dat lycopeen de IGFBP-1 concentraties bij vrouwen en de IGFBP-2 concentraties bij mannen en bij vrouwen met een verhoogd risico op dikkedarmkanker deed toenemen en daarmee mogelijk de hoeveelheid biobeschikbaar IGF-I verlaagt. Bovendien suggereren onze resultaten dat isoflavonen mogelijk alleen de circulerende IGF-I concentraties zouden kunnen verlagen bij mannen die equol kunnen vormen, die ongeveer 30-50% van de Westerse bevolking uitmaken. Beide bevindingen moeten nader onderzocht worden in toekomstige gerandomiseerde, gecontroleerde studies. De resultaten van onze studies en ander bewijs dat tot nu toe is gepubliceerd ondersteunen geen gezondheidsclaims voor lycopeen en isoflavonen in het verlagen van circulerende IGF-I concentraties bij vrouwen met een verhoogd risico op premenopauzale borstkanker noch bij mannen en vrouwen met een verhoogd risico op dikkedarmkanker. Hoewel zowel circulerende en locale IGF-systeem componenten zijn gerelateerd aan het ontstaan van borstkanker en dikkedarmkanker hebben wij geen associate gevonden tussen dikkedarmweefsel- en circulerende niveaus van deze componenten. Of IGF-I in de bloedcirculatie oorzakelijk gerelateerd is met het risico op kanker in de mens moet nader onderzocht worden.

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Alina

About the author

Curriculum Vitae

Alina Vrieling was born on July 22nd 1976 in Smilde, The Netherlands. In 1994, she completed secondary school (CS Vincent van Gogh, Assen), and started her studies in Nutrition and Health at the Wageningen University. During her studies, she developed a great interest in the role of dietary and lifestyle factors in cancer development. This was the reason for her first thesis about the relation between cigarette smoking and genetic alterations in colon tumors (Department of Human Nutrition, Wageningen University). Subsequently, she conducted a literature study on the measurement of colonic transit time, as part of a dietary and exercise intervention study (Department of Medical Physiology and Sports Medicine, University Medical Centre Utrecht). To obtain more knowledge about the mechanisms behind the role of nutrition in the development of cancer, in her third thesis she examined the influence of n-6 and n-3 polyunsaturated fatty acids on different determinants of growth in human Caco-2 colon tumor cells (Division of Toxicology, Wageningen University). Her practical period was conducted at the Division of Nutrition and Dietetics of the University of Newcastle in Australia, where she worked on a case-control study investigating the pre-illness n-6/n-3 polyunsaturated fatty acid ratio in the diet as a risk factor for inflammatory bowel disease. After her graduation in September 2000, she was involved in the development of a course on Nutrition and Health at the Wageningen University. From May 2001 until November 2001, she worked as a nutritionist at the Unilever Health Institute in Vlaardingen. In February 2002, she started her PhD-project at the Division of Experimental Therapy of the Netherlands Cancer Institute, in collaboration with the Department of Epidemiology. The work of this project is described in this thesis. Since September 2007, she is appointed as a postdoctoral epidemiologist at the Centre for Nutrition and Health of the National Institute of Public Health and the Environment (RIVM) in Bilthoven where she is engaged in the EPIC study.

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